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**The monosodium iodoacetate-induced model of  
osteoarthritis pain: behavioural, pharmacological,  
immunohistochemical and electrophysiological  
studies.**

By  
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*A thesis submitted to the University of London for the degree of  
Doctor of Philosophy*

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## Abstract

Osteoarthritis (OA), a chronic degenerative joint disease, will affect an increasing number of individuals as the population ages. It is only recently that pain, the main cause of complaint from patients, has become a source of interest. This thesis investigates a chemical model of knee osteoarthritis pain induced by intra-articular injection of monosodium iodoacetate, an inhibitor of glycolysis. After validating this model and developing new behavioural tests to assess mechanical and cooling hypersensitivity as well as ambulatory-evoked pain, the effects of existing analgesics such as morphine and gabapentin following chronic administration were studied. Both agents decreased pain behaviour but with different time-courses.

Using the same techniques as well as hindpaw weight-bearing distribution, the role of inflammation in the model was investigated using clinically available drugs: a glucocorticosteroid, methylprednisolone, a TNF (tumor necrosis factor) alpha antagonist, etanercept, and a COX (cyclo-oxygenase) inhibitor, metacam. Based on the effects of these different drugs, inflammation must play a major role during the early stages of the OA induction rather than during the later pain state.

Furthermore, *in vivo* electrophysiology studies revealed a trend towards higher excitability of deeper wide dynamic range neurones to mechanical stimuli in OA rats. Superficial dorsal horn neurokinin-1 (NK-1) receptor expressing neurones were not found to play a significant role in OA-induced behavioural changes. This indicates differences in central sensitisation and descending modulation from the brainstem, compared to other models.

A detailed study of neuronal markers in dorsal root ganglia using immunohistochemistry showed no major neuropathic pain component.

Finally, AS006, a novel peripherally selective mu-opioid receptor agonist, was shown to reduce pain behaviour.

These behavioural, immunohistochemical and electrophysiological results not only validate the use of this model for the study of osteoarthritis pain but reveal several important underlying mechanisms of nociceptive transmission in this condition.



## Acknowledgments

In December 1854, at the University of Lille, Louis Pasteur rightly claimed:

« Dans les champs de l'observation, le hasard ne favorise que les esprits préparés »,  
"In the fields of observation chance favors only the prepared mind".

My gratitude will thus first go to my supervisors, Tony and Mac, as well as to everyone I was lucky enough to meet during the four years of this great experience. Thank you for helping me to prepare my mind.

Oscar Wilde also wrote:

"Success is a science; if you have the conditions, you get the result".

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Good luck with your future professional life, your future is in your hands.

And do not forget:

*Non quia difficilia sunt non audemus, sed quia non audemus, difficilia sunt.*

"It is not because things are difficult that we do not dare,  
but because we do not dare, things are difficult."

Seneque.

### Abbreviations

ACTH	Adrenocorticotrophic hormone, corticotropin
ADP	Adenosine diphosphate
AEPS	Ambulatory-evoked pain score
AMP	Adenosine monophosphate
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BNF	British national formulary
CB1	Cannabinoid receptor 1
CamKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic AMP
cGMP	Cyclic GMP
CGRP	Calcitonin gene-related peptide
CIBP	Cancer-induced bone pain
CNS	Central nervous system
COX	Cyclo-oxygenase
CREB	cAMP response element-binding
DRG	Dorsal root ganglion
EP	Prostaglandin E receptor
ERK	Extracellular signal-regulated kinase
FB	Fast Blue
FG	Fluoro-Gold
GABA	Gamma-amino butyric acid
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
Glu	Glutamate
GMP	Guanosine monophosphate
HCl	Hydrochloric acid
IB4	Isolectin B4
IEG	Immediate early gene
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
kg	Kilogram
K <sub>v</sub>	Potassium channel

I	Litre
LRG	Late response gene
LTD	Long-term depression
LTP	Long-term potentiation
µg, µl, µm	Microgram, microlitre, micrometre
M	Molar, mol/l
MAPK	Microtubule-associated protein kinase
MHRA	Medicines and Healthcare products Regulatory Agency
MIA	Monosodium iodoacetate
min	Minute
ml	Millilitre
NaCl	Sodium chloride
NF200	Neurofilament 200
NK-A	Neurokinin-A
NK-B	Neurokinin-B
NK-1	Neurokinin-1
NO	Nitric oxide
NS	nociceptive-specific
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
PCR	Polymerase chain reaction
PD	Post-discharge
PGE2	Prostaglandin E2
PKA, PKC	Protein kinase A, Protein kinase C
PLA, PLC	Phospholipase A, Phospholipase C
qPCR	Quantitative Polymerase chain reaction
RA	Rheumatoid arthritis
SAP	Saporin
SNL	Spinal nerve ligation (L5 and L6)
Src	Tyrosine kinase
SP	Substance P
THC	Tetrahydrocannabinol
TNF-alpha	Tumor necrosis factor alpha
VGCC	Voltage-gated calcium channel
WDR	Wide-dynamic range

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## **Chapter 1. Introduction**



According to the IASP (International Association for the Study of Pain, [www.iasp-pain.org](http://www.iasp-pain.org)), pain is defined as an “unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. Acute pain is the normal, predicted physiological response to an adverse chemical, thermal, or mechanical stimulus associated with surgery, trauma, and acute illness; it is generally time-limited and is responsive to opioid therapy, among other therapies. Acute pain can sometimes lead to chronic pain, a pain state which is persistent and in which the cause of the pain cannot always be removed or is difficult to treat. Chronic pain may be associated with a long-term incurable or intractable medical condition or disease.

A recent pan-European survey embracing more than 46,000 people from 16 countries showed that chronic pain was affecting the lives of one in five adults in Europe (H. Breivik et al., 2006). Arthritis was found to be the primary cause of chronic pain being quoted by more than 30% of respondents. Osteoarthritis (OA), the most common form of arthritis (WHO, 2003; D. T. Felson, 2004), is a degenerative joint disease that is associated with chronic debilitating joint pain and affects millions of patients worldwide.

As a major cause of chronic pain, OA is thus a very relevant disease to study. Moreover, there is no widely available disease-modifying treatment available and the only alternative is to treat the symptomatic pain.

## 1.1 The joint, an organ on its own?

A joint is the location at which two bones make contact.

Structurally, joints can be classified as:

- 1) **fibrous** - bones are connected by fibrous connective tissue e.g. cranial sutures,
- 2) **cartilaginous** - bones are connected by cartilage e.g. the pubic symphyse joints,
- 3) **synovial** - there is a space called synovial cavity between the articulating bones e.g. in the knee.

Functionally, joints can be classified as **synarthrosis** - they permit no movement, **amphiarthrosis** - they permit little movement, or **diarthrosis** - they permit a variety of movements e.g. flexion, adduction, pronation. Only synovial joints are diarthrotic.

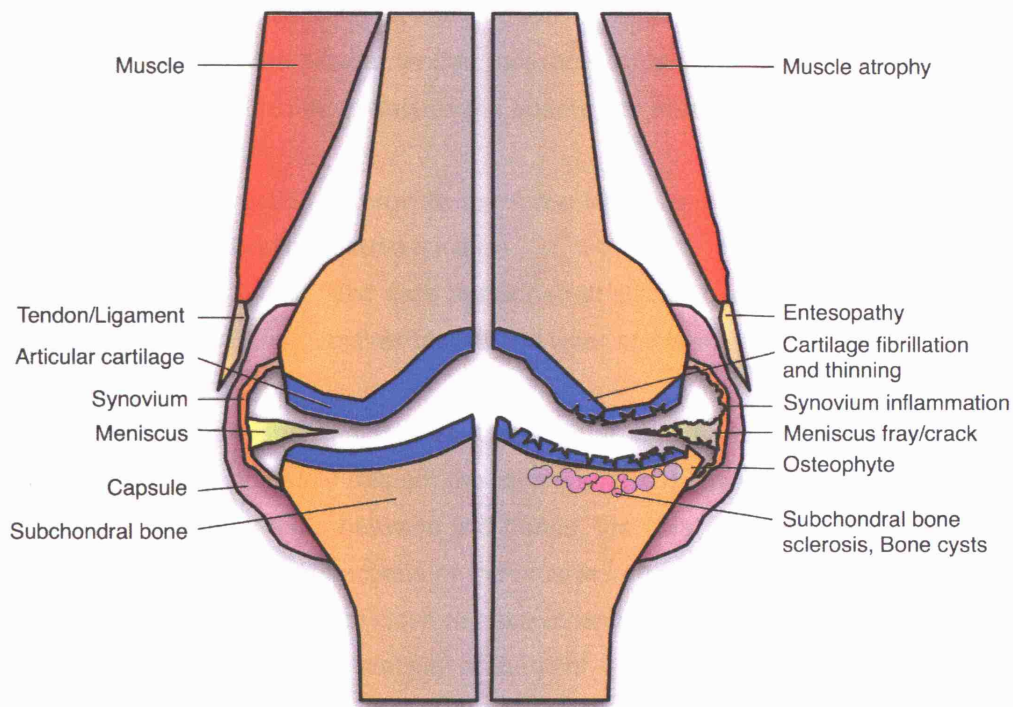


Figure 1.1. Simplified anatomy of the knee, a synovial joint. On the left, normal constituents of the joint with no fissures in cartilage and no signs of synovial inflammation. On the right, osteoarthritic knee showing early focal degenerate lesion and fibrillated cartilage, as well as remodelling of bone leading to bony outgrowth (osteophyte) and subchondral sclerosis (Adapted from H. A. Wieland et al., 2005).

Since OA is more a condition of synovial joints and so I will focus on these, especially the knee. Figure 1.1. (left) shows the basic structure of a knee joint, including:

- the articular cartilage capping the ends of bones whose principal roles are to reduce friction at the joint, to act as a cushion to absorb shocks associated with joint use, and to efficiently transmit weight loads to the underlying bone while providing a slick surface so that the bone ends can easily glide across each other during movement. Because of its importance in osteoarthritic mechanisms, its physiology will be discussed further;
- the menisci, two C-shaped pieces of cartilage, playing an important role in joint stability, lubrication, nutrition and weight-bearing distribution;
- a capsule made of a tough membrane that encloses the joint and connects one bone to another while holding them firmly in place;
- the synovium or inner lining of the capsule which secretes the synovial fluid to lubricate and nourish cartilage;
- ligaments attaching bones together and helping to provide stability;

- tendons attaching muscle to bone, allowing for movement and acting as secondary joint stabilisers, in particular the patellar tendon (not represented on Figure 1.1 for simplification purposes, see Figure 1.16) that attaches the quadriceps muscle to the tibial bone, with the patella integrated;
- some muscles contracting to provide the force for movement and that are critical for much of the shock absorption around a joint;
- some bursae or small fluid-filled sacs positioned at strategic points to cushion ligaments and tendons, protecting them against friction and wear and tear.

It is important to bear in mind that all these different structural components are not independent but are intimately related: the joint could thus be seen as an organ. Indeed, the knee joint is the interface between two bones, the tibia and the femur. Those bones can only be mobilised by contractions of the muscles attached to themselves by tendons. These two bones are further kept close to each other by the anterior and posterior cruciate ligaments that participate in the stability of the joint. The articular surface itself acts like a cushion or a sponge between the two bones: it is made of cartilage, produced by specialised cells, the chondrocytes. This articular space is also lubricated by the synovial fluid, which enables the nutrition of chondrocytes, as cartilage is not vascularised. This synovial fluid is produced by a component of the knee capsule, the synovial membrane, by filtration of the blood.

Concerning the innervation of the joint, the main structures involved are the subchondral bone – the bone directly in contact with the aneural cartilage - and the synovial capsule.

The importance of each component of the joint has been discussed; without either of them the joint cannot be fully functional. The subject matter will now shift to focus on the cartilage, mainly because of its limited regeneration properties.

## 1.2 Cartilage tissue

As for joints, different types of cartilage can be distinguished:

- **Hyaline cartilage** -also known as **articular cartilage**- covers the ends of bones within joints to allow smooth movement. It is also found in the growth plates of long bones in children;

- **Fibro-cartilage** is found in the intervertebral disc, temporo-mandibular (jaw) joint, meniscus of the knee joint, and at the site of fracture healing;
- Finally, **elastic cartilage** is found in the epiglottis (valve preventing food entering the airway) and eustachian tube (connecting the throat to the middle ear).

In the knee joint, the cartilage of interest is the **articular cartilage**. It is made of 70% water, 15% collagen, and the remaining 15% of proteoglycans (protein-glucosamine molecules), chondrocytes, non-collagen proteins, lipids and inorganic material.

Chondrocytes sit within a matrix of proteoglycans and collagen that are related in such a way as to give the cartilage its important compressive and tensile properties. This matrix provides the structural framework of the tissue and also forms a fluid compartment for transport of nutrients, waste products, chemical messengers and hormones, to and from the chondrocytes. Cartilage constantly undergoes dynamic remodelling with a balance between cartilage synthesis (through chondrocyte activity) and cartilage destruction by proteolytic enzymes such as matrix metalloproteinases (MMPs).

Cartilage is continuously bathed in the synovial fluid that is produced by the synovial membrane at the margin of the joint surface. The synovial fluid corresponds to a dialysate of plasma and also contains hyaluronic acid that enhances its viscosity. Again, as cartilage does not receive any direct blood supply, it mainly receives its nutrients from the synovial fluid that not only nourishes chondrocytes but also lubricates joint surfaces. Waste present in the synovial fluid will be removed by circulating white blood cells that can phagocytose debris.

Finally, cartilage is poorly innervated by nerve fibres, unlike the underlying subchondral bone.

### 1.3 Osteoarthritis

Osteoarthritis (OA) is a **chronic joint disease**. Unlike rheumatoid arthritis (RA), it has no systemic component. In the past, it was known as a **degenerative joint disease**, or “**wear-and-tear**” arthritis. However at the beginning of the 20<sup>th</sup> century, histopathologists and radiologists showed that degeneration in OA was also accompanied by repair. Chronic arthritis was then classified as *atrophic* or *hypertrophic*. Synovial inflammation with erosion

or atrophy of cartilage and bone was a main pattern of atrophic arthritis; however it was also associated with many other diseases including rheumatoid arthritis (RA) and septic arthritis. Hypertrophic arthritis, by contrast, was characterised by more focal cartilage loss, minimal evidence of inflammation, and by hypertrophy of adjacent bone and soft tissues; this group became synonymous with OA (J. E. Goldthwaite, 1904). However, concepts of OA evolved and according to Doherty (1994), in the 1970s OA was often used as a 'non inflammatory' disease, or even as a surrogate for normal joint tissue, hence the use of the term "osteoarthrosis" to emphasise the lack of overt inflammation. Since then, inflammation was shown to be an important component - even if it is not present all the time - and the term "osteoarthritis" is now generally used.

**One definition of OA could be: a condition of synovial joints characterised by chondropathy (cartilage loss) and evidence of accompanying periarticular bone response.**

### 1.3.1 OA diagnostics

The previous definition is easily applicable to patients and OA diagnostics depends on three quasi-independent criteria: the pathology (the study of the disease, e.g. signs of cartilage damage), the radiology i.e. any visible radiographic sign such as joint space narrowing, presence of osteophytes (bone spurs), bone sclerosis (thickening of the bone making it look whiter on X-rays), and finally the clinical symptoms such as pain, loss of movement or joint stiffness. Diagnosing OA is not a straightforward process: for example, some individuals may show osteophytes on X-rays but have no symptomatic signs of OA.

Figure 1.2.B illustrates a pathological femoral head presenting marginal osteophytes, change in bone shape, subchondral cysts and focal areas of extensive loss of articular cartilage.

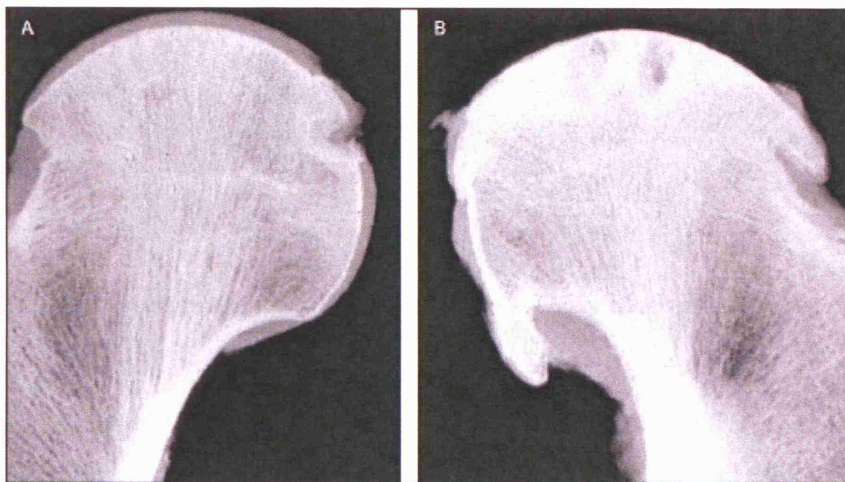


Figure 1.2. Radiograph of (A) normal and (B) osteoarthritic femoral head. From (P. A. Dieppe and L. S. Lohmander, 2005)

Based on radiographic criteria, different grades of OA can be given using the Kellgren and Lawrence scale from 0 to 4 (Table 1.1 below).

Grade 0	No OA	No features of OA
Grade 1	Doubtful OA	Minute osteophyte, doubtful significance
Grade 2	Minimal OA	Definite osteophyte, unimpaired joint space
Grade 3	Moderate OA	Moderate diminution of joint space
Grade 4	Severe OA	Joint space greatly impaired with sclerosis of subchondral bone

Table 1.1. Radiographic criteria for the assessment of OA (Kellgren and Lawrence).

Pain ratings are also important, as it is often the pain that will bring the patients to the clinics.

### 1.3.2 OA epidemiology.

Country	2002	2007	2012
United States	13.2	14.4	15.5
Europe	14.5	15.2	15.8
Japan	6.6	6.9	7.2
OA total prevalent cases	34.3	36.5	38.6
RA total prevalent cases	2.8	3.1	3.4

Table 1.2. Osteoarthritis prevalence. Number (in millions) of diagnosed total prevalent cases of OA (From H. A. Wieland et al., 2005).

As shown on Table 1.2, in 2002 it has been estimated that 14.5 million people in Europe were affected by OA, but this prevalence is projected to increase in the next decade, reaching nearly 16 million people by 2012. OA can occur in any joint, Figure 1.3 illustrates the possible joints that are affected. The most common joint affected is the knee, whereas hip, shoulder, spine and toes are less frequently affected.

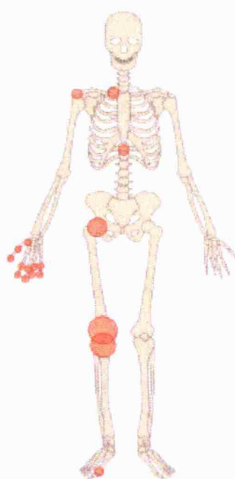


Figure 1.3. Common joints affected by OA. (H. A. Wieland et al., 2005)

No overall sex difference is obvious, although women tend to be more prevalent in terms of the severe grades of OA, in older age groups and for OA of the hand and the knee (M. Doherty, 1994).

According to the CDC (Center for disease control and prevention, USA), in 1997 the total cost of arthritis and other rheumatic conditions in the United States was 86 billion dollars including direct medical costs for 51.1 billion dollars and indirect costs (due to lost wages) for 35.1 billion dollars, which demonstrates OA huge economical impact.



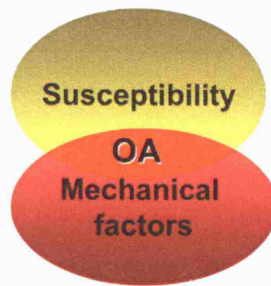


Figure 1.4. OA is the result of both susceptibility and mechanical factors

It is important to bear in mind that OA is a multifactorial disease with multiple risk factors (i.e. a factor that increases a person's chances of developing a disease). Two main categories of these risk factors can be distinguished for OA, but they are both intimately related in the development of the disease.

Susceptibility factors include age, body build (obesity), heredity (in some families, OA seems to be inherited (T. D. Spector and A. J. MacGregor, 2004)), osteoporosis, hypermobility, smoking, and other diseases. An impairment in mitochondria metabolism has also been hypothesised as a cause of OA development and progression (R. Terkeltaub et al., 2002; F. J. Blanco et al., 2004; C. Ruiz-Romero et al., 2006).

Mechanical factors could be any kind of joint trauma, joint shape and repetitive use of the joint whether it is occupational or for leisure.

The main risk factors for OA are age, obesity and any form of joint trauma. The prevalence is expected to increase because of an aging population. However, many old people will not develop OA, which suggests that it is not a universal feature of ageing.

### 1.3.3 *Pathophysiology of OA*

Focal cartilage destruction is one of the key features of OA. As noted earlier, under normal conditions, there is equilibrium between cartilage synthesis and cartilage degradation. One of the first pathological signs of OA consists of fissuring of the articular cartilage. Progressive focal erosion and ulceration can appear at some sites, along with cell necrosis; cartilage repair also occurs through chondrocyte proliferation at adjacent sites but can only lead to the formation of disordered reparative cartilage. Changes in the subchondral bone then appear, with fissuring and microfractures with, at advanced stages, the formation of cysts. Consequently, 'new' subchondral bone - causing bone sclerosis - and osteophytes are formed. In the meantime, various proteases such as collagenases of



the MMP family, as well as some inflammatory mediators are released in the synovial fluid, which can also contribute to cartilage damage.

## **1.4 OA pain**

Clinical OA and pain are often associated. Under these circumstances, pain can be seen as a protective mechanism, which leads to the question: is it really beneficial to relieve pain in patients suffering from OA? What consequences does pain relief have on the joint structural changes? Is pain just accompanying the structural changes? Arguably, this is why research was focussed on structural changes associated with the disease rather than OA pain. However, once those changes i.e. the late features of OA are visible, it is then already too late, and the only solution consists of trying to stabilise the cartilage condition. Ideally, identifying some early markers of OA would be a great improvement in the management of the condition. Until then, pain still has to be treated and it is present in patients' everyday life. Thus the discussion will try to illustrate how decreasing pain can help in improving OA patients' life.

### **1.4.1 Prevalence**

As was stated earlier, arthritis was shown to be the first cause of chronic pain in a pan-european survey (H. Breivik et al., 2006). While the back is the first body location for pain (42% of respondents), the knee comes second, affecting 16% of respondents.

When the patients were asked to quote the cause of their pain, arthritis and osteoarthritis was quoted by 34% of respondents, making it the first cause of chronic pain. OA pain is therefore a very important area with unmet needs.

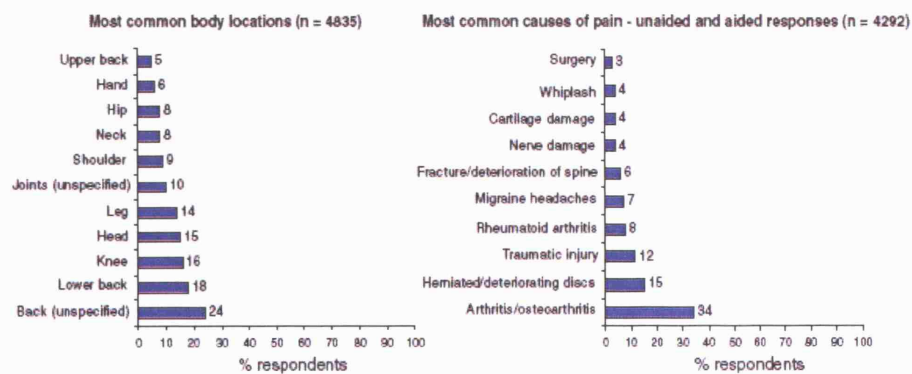


Figure 1.5. Body location and causes of pain: results from a pan-european survey (H. Breivik et al., 2006).

## 1.4.2 Aetiology

### 1.4.2.1 Different types of pain

Pain is commonly classified as:

- **Nociceptive**: the physiological, generally acute pain sensation evoked by direct stimulation of pain fibres, by a mechanical stimulus for example;
- **Inflammatory**: the release of inflammatory mediators following tissue damage that can directly stimulate the primary afferent fibres and thus trigger pain;
- **Neuropathic**: following the IASP definition, neuropathic pain can be caused by a nerve damage or dysfunction in the peripheral and/or central nervous system.

However some conditions do not fit in a single category. For example cancer-induced bone pain and OA pain are a mixture of different pains, which could explain why they are even more difficult to treat.

### 1.4.2.2 OA Pain

The causes of OA pain are not fully understood. Contrary to cartilage that is aneural, subchondral bone, periosteum, synovium, ligaments and the joint capsule are all richly innervated and contain nerve endings that could be the source of nociceptive stimuli in OA (G. Blackburn-Munro, 2004).

Different pain mechanisms have been suggested (M. Doherty, 1994; H. A. Wieland et al., 2005):

- stimulation of nociceptive fibres and mechanoreceptors in the capsule by intra-articular hypertension consequent upon synovial hypertrophy and increased fluid production;
- stimulation of subchondral and periosteal (the periosteum is an envelope of fibrous connective tissue that is wrapped around the bone in all places except at joints - protected by cartilage) nerve fibres by intra-osseous hypertension;
- inflammation of the synovial lining of the knee capsule;
- perception of subchondral microfractures;
- painful enthesopathy (disease occurring at the site of attachment of muscle tendons and ligaments to bones or joint capsules) and bursitis (inflammation of bursae, or bursal synovitis) that accompany structural alteration, muscle weakness and altered usage;
- alteration in the function of the surrounding musculature.

Additionally, chronic pain in OA patients is dependent primarily on the activation of primary afferents innervating the joint (P. Creamer et al., 1996). All the mechanisms quoted above, as well as others, could then lead to the peripheral sensitisation of nociceptors, thus increasing the input produced by noxious stimuli towards the spinal cord. Central sensitisation could then be induced in the spinal cord and higher centres, resulting in an amplification of pain sensation (B. L. Kidd and L. A. Urban, 2001; H. G. Schaible et al., 2002). Finally, a neuropathic component of OA pain has also been hypothesised, although no alteration in the sensory neurones has been shown and OA symptoms are quite different from neuropathic pain.

Table 1.3 shows the differences between inflammatory and neuropathic pain. OA pain might have characteristics that encompass both types of pains.

	Inflammatory pain	Neuropathic pain
<b>Precipitating cause</b>	Activation of innate and acquired immunity	(Peripheral) Nerve injury
<b>Signs and symptoms</b>	Mechanical and thermal hyperalgesia	Mechanical and thermal hyperalgesia, Cold allodynia
<b>Peripheral pathophysiology</b>	Primary afferent sensitisation	Ectopic activity
<b>Altered gene expression</b>	Transmitters/modulators	Ion channels
<b>Central pathophysiology</b>	Central sensitisation	Central sensitisation, glial activation
<b>Pharmacological sensitivity</b>	Opiates, NSAIDs	NSAIDs, Channel blockers, Decreased to opiates

Table 1.3. Comparison between Inflammatory and neuropathic pain. From Pr SB McMahon.

It is important to note the definitions of **hyperalgesia**, corresponding to exaggerated nociceptive responses to noxious stimulation, and **allodynia** corresponding to nociceptive responses to normally innocuous stimulation.

### 1.4.3 Symptoms in OA patients

Pain is often the first source of complaint from patients. However, a correlation between pain and radiographic changes varies depending on the site of OA, being best at the hip then the knee, and poorest for the extremities, although patients with the most severe X-ray changes are more likely to have symptoms than those with mild changes. As pain has both sensory and affective components, other factors such as personality, anxiety and depression may be of great influence on patients' quality of life, especially in those chronic pain states, whether it is primarily neuropathic pain (K. Meyer-Rosberg et al., 2001) or OA pain (F. Salaffi et al., 2005).

Joint stiffness is another symptom described by many patients after a period of inactivity. Joint swelling deformity and coarse crepitus (creaking) can also occur, even in the absence of pain.



Figure 1.6. L'homme, René Descartes, c. 1664. Pain is not simply transmitted to the brain from the periphery through nerve afferents as described Descartes in the XVII<sup>th</sup> century. It has two components – sensory and emotional. The latter will influence the brain's ratings of the pain, depending on other factors such as depression, anxiety, fear...

Again, it is interesting to note as with many chronic pain states, modification of mood, such as anxiety and depression are observed; furthermore fMRI showed activation of brain areas involved with fear and emotions in arthritic patients, which highlights the important role of the emotional component of pain in humans (B. Kulkarni et al., 2007). Obviously, this could have serious implications in assessing the effects of a new drug in OA patients, as pain ratings could vary between individuals because of these variable factors.

## 1.5 Nociceptive transmission

Nociceptive signals reach the brain from the peripheral site of injury via a number of parallel neuronal pathways involving sensory fibres.

### 1.5.1 *Nociceptive fibres*

Primary somatosensory afferents can be classified according to their diameter and conduction velocity. In order of decreasing diameter and conduction velocities (cf. Table 1.4), there are:

- thickly myelinated A-beta fibres,
- thinly myelinated A-delta fibres,
- unmyelinated C fibres.

	A-beta fibre	A-delta fibre	C fibre
Conduction velocity (m/s)	7 - 75	2 - 7	0.5 – 1.5
Myelination	Heavy	Light	None
DRG cell body diameter (mm)	45 - 51	33 - 38	20 - 27
Stimulus conveyed	Non-noxious	Non-noxious/ noxious	Noxious

Table 1.4. Characteristics of the different types of sensory cutaneous primary afferent fibres in adults rats (J. Schouenborg, 1984; R. S. Scroggs and A. P. Fox, 1992; A. J. Fox, 1999).

The nociceptive fibres are mainly A-delta and C fibres, while under normal circumstances A-beta fibres are associated with mechanotransduction. C-fibres are mainly polymodal nociceptors that respond to multiple forms of noxious stimulation including thermal, mechanical and chemical. In response to noxious stimuli, these somatosensory afferent fibres will produce action potentials that will be conducted to the dorsal horn of the spinal cord, with A-delta fibres mostly terminating in laminae I, II and V (according to Rexed's classification) and C fibres mainly in laminae I and II. They can then synapse with superficial nociceptive (NS) specific neurones or deep wide-dynamic range (WDR) neurones (Figure 1.7).

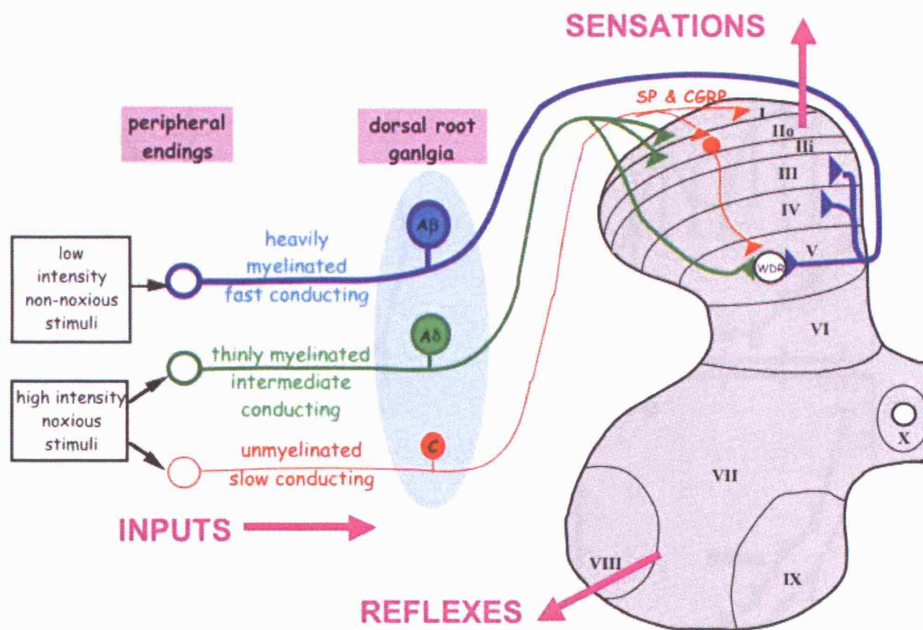


Figure 1.7. Somatosensory afferent fibres properties, from the periphery to the central nervous system. The nociceptive fibres synapse in the dorsal horn, in both superficial and deep laminae, releasing different neurotransmitters such as substance P (SP) and CGRP (Calcitonin gene-related peptide). These neurones project in turn to higher centres to produce sensations. The ventral horn mainly consists of motoneurons involved in reflex responses for example.

### 1.5.2 Primary and secondary hyperalgesia

First described as an increased pain sensation, the IASP definition recommended the use of hyperalgesia as an increased pain sensation to a stimulus that is normally painful, and allodynia for pain induced by stimuli that are not normally painful. Primary hyperalgesia corresponds to increased pain sensitivity at a site of tissue damage, whereas secondary hyperalgesia is the result of increased pain sensitivity on normal skin surrounding a site of tissue damage (Figure 1.8).

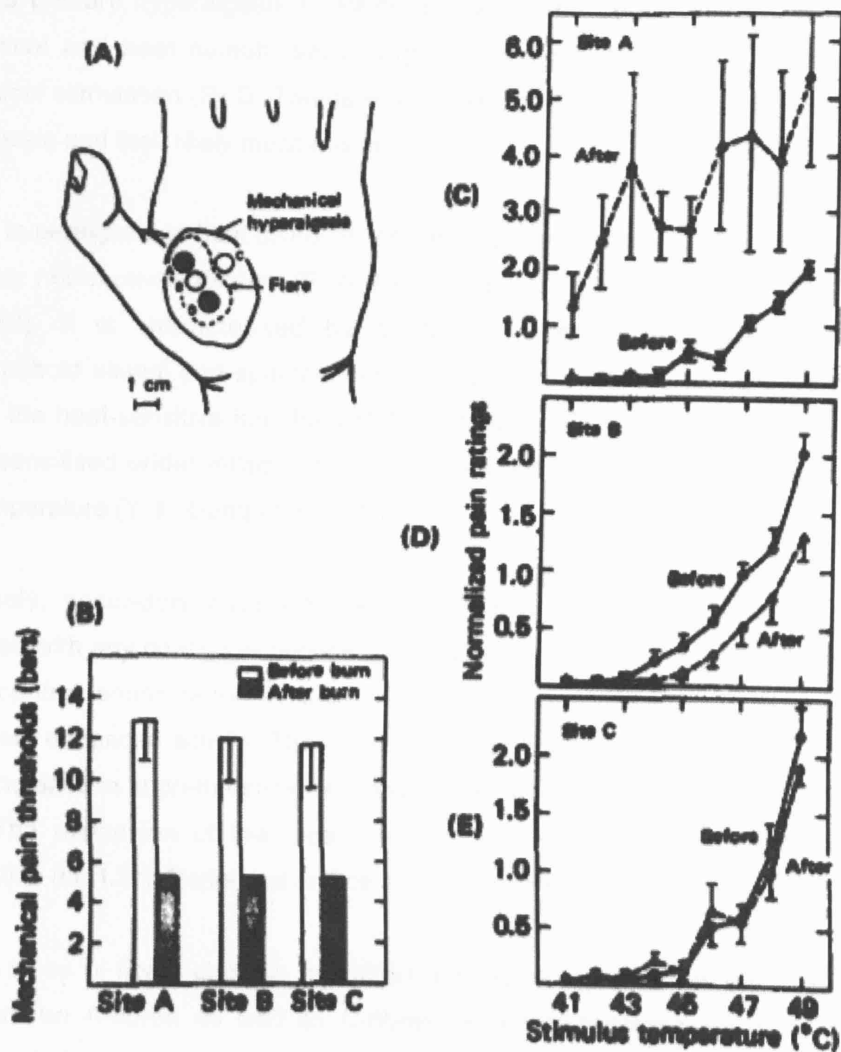


Figure 1.8. Sensory characteristics of hyperalgesia following burns to the glabrous skin. Hyperalgesia at the site of the burn (primary hyperalgesia) was characterised by hyperalgesia to heat and mechanical stimuli, whereas hyperalgesia in the uninjured tissue adjacent to the burn (secondary hyperalgesia) was characterised by hyperalgesia to only mechanical stimuli. (A) A burn injury was applied to two locations (sites A and D). Mechanical pain thresholds and ratings of pain to heat stimuli were recorded at one of the burn locations (site A), at the uninjured skin between the two burns (site B), and at an adjacent uninjured location (site C). The areas of flare and mechanical hyperalgesia in a typical subject are also shown. (B) The mean mechanical pain threshold was significantly decreased at all sites following the burns ( $n = 7$ ). Means and S.E.M. (C-E) Mean normalised pain ratings to randomised heat stimuli before and after the burns. (C) At the site of a burn, all the characteristics of heat hyperalgesia (decrease in threshold, increased pain to suprathreshold stimuli, spontaneous pain) were present after the burns ( $n = 8$ ). (D) Between the two burn sites, pain ratings decreased after the burn, indicating heat hypoalgesia ( $n = 9$ ). (E) In the area of secondary mechanical hyperalgesia (site C), heat pain ratings before and after the burns were not significantly different ( $n = 8$ ) (Adapted from S. N. Raja et al., 1984; From R. D. Treede et al., 1992).



Whereas primary hyperalgesia is mainly characterised by increased sensitivity to both mechanical and heat stimuli, secondary hyperalgesia seems to be more specific to mechanical stimulation (R. D. Treede et al., 1992). Table 1.5 shows the different types of hyperalgesia and their likely mechanisms.

Primary hyperalgesia to heat stimuli is the result of peripheral sensitisation of the terminals of primary nociceptive afferents (R. A. Meyer and J. N. Campbell, 1981; R. H. LaMotte et al., 1982). It is characterised by decreased threshold, increased responses to suprathreshold stimuli and spontaneous activity. These primary nociceptive afferent also express the heat-sensitive ion channel TRPV1 (M. J. Caterina and D. Julius, 2001) that can be sensitised under inflammatory conditions, which can lead to activation at normal body temperature (Y. F. Liang et al., 2001).

Conversely, secondary hyperalgesia in response to mechanical stimulation is not associated with any change in peripheral coding (T. K. Baumann et al., 1991) but is rather due to central sensitisation of second order neurones in the spinal cord and their subsequent enhanced activity. These neurones display a drop in their activation threshold and an increase in suprathreshold responses after a peripheral insult (D. A. Simone et al., 1991). The expansion of the receptive field is another prominent feature of central sensitisation (cf. 1.5.5. Peripheral and central sensitisation).

Different types of fibres underlie the different types of hyperalgesia. Heat hyperalgesia is dependent on A-fibres as well as C-fibres. Punctate hyperalgesia, both primary and secondary, is believed to rely on A-fibre nociceptors. Stroking hyperalgesia would be more likely to depend on A-beta fibres.

Finally, the mechanisms of cold hyperalgesia, frequent in neuropathic pain patients, are not well characterised. Peripheral sensitisation cannot be ruled out as the peripheral encoding of noxious cold stimuli have not been investigated extensively. Other mechanisms include central sensitisation or a central disinhibition by selective loss of a sensory channel specific for non-noxious cold that could exert a tonic inhibition on nociceptive channels (A. D. Craig and M. C. Bushnell, 1994).

Test stimulus	Occurrence	Afferents	Sensitization
heat	primary zone	type I & II AMH, CMH	peripheral
blunt pressure	primary zone	MIA, (type I AMH?)	peripheral
impact	primary zone	MIA, (type I AMH?)	peripheral
punctate	neuropathic secondary zone primary zone	type I AMH type I AMH type I AMH, MIA	central central peripheral/central?
stroking	neuropathic secondary zone primary zone	A $\beta$ -LTM A $\beta$ -LTM A $\beta$ -LTM	central central central
cold	neuropathic pain secondary zone?	? ?	central? central?
chemical	inflammation	type II AMH, CMH, MIA ?	peripheral ?

Table 1.5. Different types of hyperalgesia and their likely mechanisms. Abbreviations: **A-beta-LTM** A-beta-fibre low-threshold mechanoreceptor ("touch receptor"), probably rapidly adapting subtype (Meissner corpuscle); **type I AMH** A-fibre nociceptor with slow high-threshold heat response (no TRPV1), probably equivalent to A-fibre high-threshold mechanoreceptor; **type II AMH** A-fibre nociceptor with rapid low-threshold heat response (TRPV1); **CMH** C-fibre mechano-heat nociceptor (TRPV1); **MIA** mechanically insensitive (silent) nociceptive afferent (From R. D. Treede et al., 2004).

### 1.5.3 Neurotransmitters and receptors involved in spinal nociceptive transmission

#### 1.5.3.1 Neurokinins

The tachykinin or neurokinin peptide family includes substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) that can interact with three neurokinin receptors, NK-1R, NK-2R and NK-3R. Substance P, NKA and NKB exhibit the highest affinity for NK-1R, NK-2R and NK-3R respectively, but each ligand can activate the other receptor types when present at a sufficiently high concentration. These receptors are G protein-coupled receptors (GPCRs), all of which can activate both phosphatidylinositol hydrolysis and the cAMP signal cascade (Y. Nakajima et al., 1992). In pain transmission, SP is the most important member of the family. Indeed, dorsal horn SP has been shown to originate from primary afferent fibres and intrinsic neurones, together with a contribution from descending fibres. The neurokinin receptors have been shown to be mainly postsynaptic to the afferent fibre terminals and to be located in laminae I, II and X of the spinal cord (M. Otsuka and K. Yoshioka, 1993). The release of SP following a noxious stimulation has been shown in many studies (A. W. Duggan et al., 1988). Moreover, both SP and NKA levels have been shown to increase after peripheral inflammation. It has also been demonstrated that after noxious but not after non-noxious somatic stimulation, there was a release of SP in the

spinal cord (released presumably primarily from SP-containing primary afferent fibres), which in turn induced the internalisation of SP receptors in lamina I spinal neurones expressing that receptor (C. Abbadie et al., 1997; P. Honor et al., 1999). In addition, under somatic inflammatory conditions, there seems to be an increased ventral diffusion of SP from primary afferents because the SP receptor-expressing neurones located in laminae III and IV also internalised the SP receptors after noxious stimulation.

All in all, these results suggest that after somatic inflammation, there is substantial neurochemical plasticity so that an increased number of SP receptor-expressing neurones can respond to normally non-noxious and noxious stimulation. SP receptor-expressing neurones in the spinal cord appeared to play a significant role in the ascending conduction of both somatic inflammatory and neuropathic pain. As a result, although only representing 5 to 10% of the total number of lamina I neurones, nearly all of those SP receptor expressing neurones projected to higher areas of the brain involved in nociceptive signalling. These lamina I NK-1R-expressing neurones were shown to receive substantial input from SP-releasing sensory C-fibres and to terminate within the thalamus and parabrachial area of the brain (Y. Q. Ding et al., 1995; G. E. Marshall et al., 1996; A. J. Todd et al., 2000). Neurones within that part of the parabrachial area usually responded to most types of noxious (but not non-noxious) stimulation, were intensity coded, and had large receptive fields that could include the whole or one side of the body (C. A. Doyle and S. P. Hunt, 1999; H. Bester et al., 2000). Ablation of those neurones with the neurotoxin conjugating SP and saporin (SAP) resulted in a decrease in somatic inflammatory and neuropathic pain. Antagonists of the tachykinin receptors were not fully characterised but NK-1 and NK-2 receptors may have roles not in acute pain but more in central hypersensitivity pain states (T. Yamamoto and T. L. Yaksh, 1991; V. Chapman and A. H. Dickenson, 1992; X. J. Xu et al., 1992). The spinal release of these peptides was also shown to increase spinal cord excitability after inflammation for example (K. A. Sluka et al., 1992; J. D. Levine et al., 1993).

### 1.5.3.2 CGRP

CGRP, or calcitonin gene-related peptide, is released by primary afferents. It is known to be released after noxious stimulation and to excite dorsal horn neurones, but its role in pain processing is not well known yet.

Immunohistochemistry has shown that CGRP is expressed in approximately 45-70% of lumbar DRG neurons (P. W. McCarthy and S. N. Lawson, 1990; V. M. Verge et al., 1993). According to conduction velocity studies, most of CGRP-containing neurones could be classified as nociceptive as it was expressed in 46% of the C fibres - the so-called peptidergic C fibres, 33% of the A-beta fibres and 17% of the A-delta fibres (P. W. McCarthy and S. N. Lawson, 1990).

CGRP is released from the spinal terminals of primary afferent neurons by high-intensity mechanical and thermal stimuli as well as by the injection of local irritants (C. R. Morton and W. D. Hutchison, 1989; M. G. Garry and K. M. Hargreaves, 1992).

CGRP effects are believed to be mediated by the calcitonin-like receptor that is a G<sub>s</sub>-coupled seven-transmembrane spanning receptor (D. L. Hay et al., 2004).

#### **CGRP and pain**

CGRP application serves to induce spinal facilitation of the dorsal horn response which was blocked by putative CGRP antagonism (R. Q. Sun et al., 2003). Ionophoretic application of CGRP has been shown to induce a potentiation of the depolarising effects of SP (G. Biella et al., 1991).

CGRP might also be involved in migraine pain. It has been shown that the CGRP antagonist BIBN 4096 BS significantly reduced acute pain in migraine, by acting on the neurogenic inflammation caused by the release of vasoactive neuropeptides such as CGRP from nociceptive perivascular nerve (M. K. Herbert and P. Holzer, 2004).

In animal studies, intrathecal delivery of partial CGRP sequences, believed to be antagonistic, resulted in reduction in the hyperalgesia induced by intradermal capsaicin (R. Q. Sun et al., 2003). Spinal delivery of CGRP8-37, a CGRP antagonist, has been reported to increase thermal escape latency following inflammation and neuropathy (L. C. Yu et al., 1996b; L. C. Yu et al., 1996a) as well as to diminish the writhing response induced by phenylbenzoquinone (M. A. Saxen et al., 1994), thermal hyperalgesia and tactile allodynia otherwise observed after cord hemisection (A. D. Bennett et al., 2000). Additionally, tumours from hyperalgesic mice were shown to be more densely innervated with calcitonin gene-related peptide (CGRP)-immunoreactive nerve fibres and less densely vascularised than tumours from non-hyperalgesic mice (P. W. Wacnik et al., 2005).

**Relevance to OA**

CGRP, along with substance P, was also found to be present in nerve terminals in the synovium of osteoarthritis patients' knees (T. Saito and T. Koshino, 2000) and was increased after OA induction with MIA in rats (J. Fernihough et al., 2005). These studies tend to suggest a more peripheral role of this peptide.

**1.5.3.3 Somatostatin**

Somatostatin is expressed in small diameter cells in the dorsal root ganglion (DRG) and afferent terminals in the *substantia gelatinosa* (lamina II) of the spinal cord. It is suggested that it may have an inhibitory role in the dorsal horn. However, it may not be used as an analgesic yet, as the side-effects are produced at doses very close to the antinociceptive ones (P. Mollenholt et al., 1988).

**1.5.3.4 Galanin**

This peptide colocalises with SP and CGRP in a large proportion of primary afferent neurones. It is thought to have antinociceptive effects but further studies are necessary to determine the role of that peptide in pain transmission. However a decreased spinal cord sensitisation to C-fibre stimulation was demonstrated in mice overexpressing galanin (S. Grass et al., 2003).

**1.5.3.5 Excitatory alpha-amino acids**

Excitatory alpha-amino acid transmitters account for most of the fast synaptic transmission that occurs in the mammalian brain. Glutamate and aspartate are the major excitatory amino acid neurotransmitters. However, aspartate does not seem to fulfil all the criteria of a neurotransmitter, which is highlighted by the fact that no reuptake mechanism nor rapid characterised release mechanisms are known.

The excitatory effects of L-glutamate were first observed more than 40 years ago, and since that time it has been demonstrated conclusively that L-glutamate is the predominant excitatory neurotransmitter in the CNS. Glutamate is the transmitter at more than 95% of excitatory synapses and, therefore, glutamatergic neurotransmission is involved in nearly

every aspect of CNS function. It is present in myelinated and unmyelinated primary afferent fibres in addition to intrinsic interneurons and projection neurons.

Different kinds of receptors known to be activated by excitatory amino acids are believed to play a role in pain processing. They are divided into two groups: the N-methyl-D-aspartate (NMDA) receptor and the non-NMDA receptors. The non-NMDA receptors consist of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), the metabotropic (mGlu) and the kainate.

The coexistence of both glutamate and substance P (SP) in more than 90% of the SP containing fibres (G. Battaglia and A. Rustioni, 1988) rose great interest as it is very likely that both excitatory amino acids and peptides are released by afferent fibres following a noxious stimulation.

The action of the amino acids on the NMDA receptor complex and the postsynaptic events in the spinal cord has thus been the centre of interest. The NMDA receptor has been implicated in a number of long-term events in the CNS such as in long-term potentiation (LTP) in the hippocampus, in synaptic plasticity in the visual cortex, epileptic activity and in sustained motor activity (G. L. Collingridge and W. Singer, 1990; P. M. Headley and S. Grillner, 1990; N. W. Daw et al., 1993).

Activation of the NMDA receptor, mainly by repeated C-fibre activity, requires the release of glutamate but also the presence of glycine and the means to remove the physiological levels of  $Mg^{2+}$  that normally block the channel. Indeed, this physical block of the channel means that only a sufficient depolarisation of the membrane can unblock the channels and thus enables ions to flow through them. The binding of tachykinins to neurokinin receptors that leads to a slow summing depolarisation causes this block removal. Once the channel is open, a massive depolarisation is caused by a  $Ca^{2+}$  ion influx into the neurone, leading to a sudden increase in activity, known as the wind-up, as shown in Figure 1.9. The activation of the NMDA receptor after a stimulus involving a sustained C-fibre activity or just if its frequency or intensity is enough, could thus result in the amplification or prolongation of the response and underlie many forms of central hyperalgesia (A. H. Dickenson and A. F. Sullivan, 1990; R. Dubner and M. A. Ruda, 1992; S. B. McMahon et al., 1993; A. Dray et al., 1994).

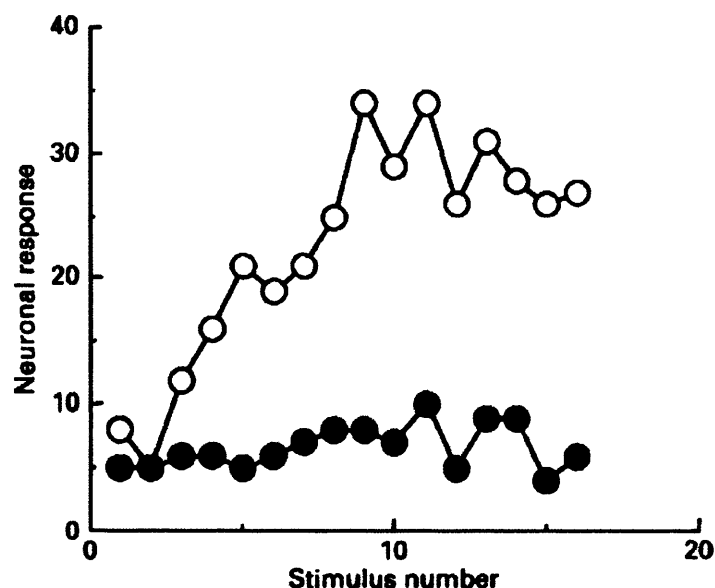


Figure 1.9. Example of wind-up in dorsal horn nociceptive neurone. A number of repeated identical C-fibre stimuli were given and the neuronal response measured in halothane-anesthetised rats. The white dots represent the control response that increases with the stimulus number. The black dots correspond to the response after administration of a NMDA antagonist, which results in the absence of wind-up (A. H. Dickenson, 1995).

It is very interesting to add that ketamine, widely used in short-term anaesthesia for minor surgery in animals is a NMDA receptor antagonist. Indeed, in addition to the magnesium-binding site in the channel pore, the NMDA receptor contains numerous other modulatory sites. Glycine is required to bind the receptor for channel activation. Glycine thus serves as a co-agonist with glutamate. Normal extracellular glycine levels are sufficiently high so that the glycine binding site on the NMDA receptor is at least partially occupied. NMDA receptors also contain modulatory sites for polyamine binding, zinc binding, and binding sites for dissociative anaesthetics such as phencyclidine (PCP) and ketamine.

#### 1.5.3.6 Nitric oxide and prostanoids

The role played by nitric oxide (NO) as a neuronal messenger was widely publicised in 1992 when NO was declared to be Molecule of the Year by the journal *Science*. NO is a gas that is soluble in the saline fluids that bathe cells and make up the blood. It is an electrically neutral, highly reactive free radical. NO is a novel neurotransmitter because it is not stored in cells and it diffuses through membranes, unlike conventional chemical neurotransmitters which are synthesised and then stored in vesicles before they are released. The diverse physiological signalling functions of NO include vasodilatation,

vasoprotection, secretory control, intestinal relaxation, penile erection, macrophage cytotoxicity, regulation of developmental processes, neuroprotection, neurotoxicity and, not least, neurotransmission and neuromodulation. Due to its novelty and importance, NO signalling is intensively studied in mammals (largely rodents) and often reviewed. Its role as a neurotransmitter is firmly established at certain synapses between identified neurones of invertebrates, and in the mammalian peripheral nervous system at nonadrenergic–noncholinergic synapses onto smooth muscle. There is substantial evidence for the role of NO as a neurotransmitter and neuromodulator in the mammalian central nervous system (CNS) as well (O. Arancio et al., 1996). For example, it participates as a retrograde transmitter in some forms of hippocampal long-term potentiation (LTP), and as an orthograde neurotransmitter–modulator in cerebellar long-term depression (LTD), in which NO stimulates cGMP production and the downregulation of glutamate receptors.

NO also appears to have a role during prolonged chronic pain states that are associated with NMDA-receptor activation. Indeed, the activation of the NMDAR followed by the  $\text{Ca}^{2+}$  influx results in the activation of the NO synthase (NOS). The NOS antagonist L-NAME abolishes facilitated reflexes, inhibits thermal hyperalgesia in neuropathic animals and the response of single dorsal horn neurones to a peripheral injection of formalin (H. J. McQuay and A. H. Dickenson, 1990). The synthesis of NOS inhibitors lacking hypertensive effects have already proved to be antinociceptive, suggesting possible therapeutic uses of NOS inhibitors (P. K. Moore et al., 1993). The proposed action of NO is as a retrograde transmitter feeding back from spinal neurones onto pre-synaptic sites to further increase transmitter release (L. S. Sorkin, 1993).

This positive feedback may also be caused by the spinal generation of prostanoids, following both NMDA and SP-induced activation of neurones, leading to the reduction of hyperalgesia when using non steroidal anti-inflammatory drugs (NSAIDs), although the relative importance of the central action compared to the systemic one of these latter is not known yet (A. B. Malmberg and T. L. Yaksh, 1992).

#### 1.5.3.7 Serotonin

Serotonin (5-HT, 5-hydroxytryptamine, synthesised from L-tryptophan) is found in all human tissues and body fluids. It can modify a variety of biological and behavioural functions, including sex, aggression, mood, appetite, locomotor activity, learning and



memory, sleep, arousal and hormonal secretion, and it is implicated in a variety of human diseases such as migraine, hypertension, depression, anxiety, schizophrenia, eating disorders and vomiting. Many of the current treatments for such disorders interact with the 5-HT system.

Serotonergic cell bodies are clustered predominantly in the midline or raphe region of the upper brainstem and pons, with extensive projections to all areas of the brain and to the spinal cord (M. M. Niblock et al., 2004).

There are different types of human 5-HT receptors found both in the central and peripheral nervous system as well as in a number of non-neuronal tissues such as the gut, cardiovascular system and blood cells. They belong to either the guanine nucleotide-binding (G) protein-coupled (metabotropic) or the ligand-gated ion channel (ionotropic) superfamilies of receptors (D. Hoyer et al., 1994).

The 5-HT<sub>3</sub> subtype receptors, the only known member of the 5-HT ionotropic receptors superfamily, are of interest to us because they are known to perform a pronociceptive function in the spinal cord, in particular through descending facilitatory pathways from the brainstem to the spinal cord (R. Suzuki et al., 2002).

5-HT<sub>3</sub> receptor antagonists such as ondansetron, granisetron and tropisetron are used to treat chemotherapy- and radiotherapy-induced nausea and vomiting.

#### 1.5.4 *Nociceptors and nociceptive transmission*

When looking more closely to nociceptors, or specialised primary afferent fibres encoding noxious stimuli, various receptors have been identified. Figure 1.10.A. shows the vanilloid receptors (VR) activated by capsaicin, heat and ATP, acid-sensing ion channels (ASIC), prostaglandin E receptors (EP) activated after inflammation by prostaglandins (PG) following cyclo-oxygenases (COX) activation and/or induction. In addition to Ca<sup>2+</sup>, Na<sup>+</sup> and K<sup>+</sup> channels, other channels like the TRP family (transient receptor potential) encode thermal stimuli, while the P<sub>2</sub>X receptors can respond to ATP.

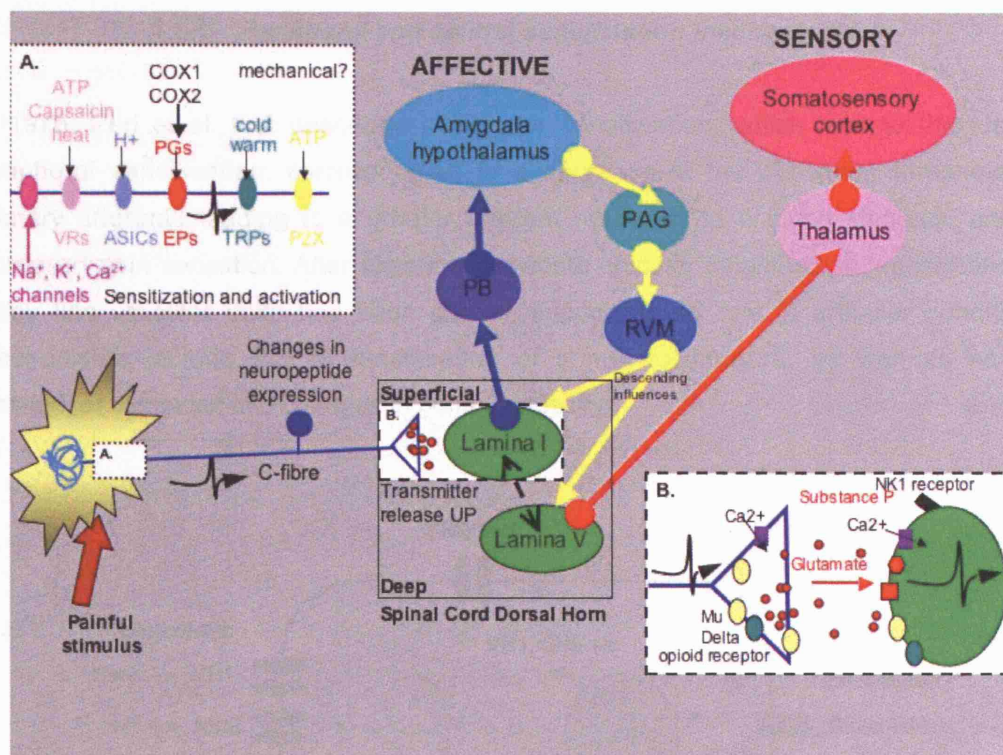


Figure 1.10. Pain transmission and processing. When a noxious stimulus is encoded by various receptors in the periphery (A), nociceptive primary afferent fibres such as C fibres conduct information to the dorsal horn of the spinal cord. From there, in response to neurotransmitter release such as SP and glutamate (B), two main neuronal populations will be activated: the superficial lamina I neurones, most of which are nociceptive specific (NS); and the deep lamina V ones, mainly wide dynamic range (WDR). These neurones then project to higher centres, activating in turn different descending pathways. PB: parabrachial area, PAG: periaqueductal grey, RVM: rostro-ventral medulla. A. Various receptors encoding noxious stimuli. VR: vanilloid receptor, ASIC: acid-sensing ion channel, EP: prostaglandin E receptor, PG: prostaglandin, COX: cyclo-oxygenase, TRP: transient receptor potential, P2X: purinergic receptor. B. Different actors in pain transmission in the spinal cord dorsal horn.

It has been known for a long time that tissue injury and inflammation often result in hyperalgesia, allodynia and spontaneous pain, which can be explained by sensitisation of the nervous system.

As shown on Figure 1.10, these spinal neurones will then project to the brain through the spinothalamic and spinoparabrachial pathways, which constitute the sensory and affective components of pain, respectively (See Review by M. J. Millan, 1999). A descending facilitatory serotonergic pathway originating in the rostro-ventromedial medulla was also shown by Suzuki et al. (2002), suggesting the existence of a spino-cerebro-spinal loop modulating pain.

### 1.5.5 Peripheral and central sensitisation mechanisms

In 1976, Perl et al. first described nociceptor sensitisation, which led to the idea of peripheral sensitisation, corresponding to a decrease in the activation thresholds of primary afferents leading to a greater afferent response to a given stimulus, and an increased pain sensation. After experimental acute articular inflammation, sensitisation of many fine articular units has been shown, supporting its role in articular conditions. Mechanisms include actual sensitisation of some nociceptors, as well as second messenger cascades and changes in gene expression.

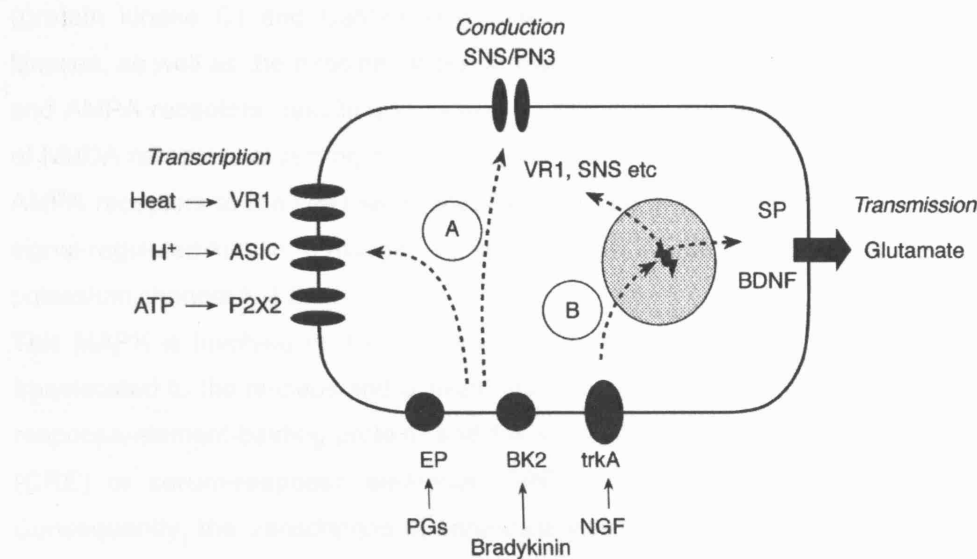


Figure 1.11. Peripheral sensitisation mechanisms following inflammation (modified from C. J. Woolf and M. Costigan, 1999; From B. L. Kidd and L. A. Urban, 2001)

For example following the early stages of inflammation, prostaglandins (PGs) and bradykinin will be released, which can cause a decrease of activation thresholds for conducting-ion channels such as VR1, ASIC or P2X (Figure 1.11, A). Transcriptional events mediated by various cytokines (interleukins, TNF...) and growth factors (NGF e.g.) can occur in the long-term (Figure 1.11, B), which will result in an increased expression of receptors, ion channels and neurotransmitters.

As shown on Figure 1.10.B, various neurotransmitters can be released in response to a pain stimulus, mainly glutamate binding to different receptors such as the NMDA, AMPA and metabotropic glutamate (mGlu) receptors for glutamate, and substance P binding to

the NK-1 receptor. Calcium channels also play a major role on neurotransmitter release and neurone excitability.

Similar to what happens in the periphery, in 1983 Woolf described central sensitisation of the dorsal horn, an increase in the excitability of the central nervous system, by testing the flexion reflex following the induction of an injury (C. J. Woolf, 1983). Figure 1.12 displays some mechanisms involved in the induction of central sensitisation. Briefly, following the release of neurotransmitters such as glutamate and substance P, binding to AMPA & NMDA and NK1 receptors respectively, the intracellular  $\text{Ca}^{2+}$  concentration increase will trigger the activation of various protein kinases, including PKA (protein kinase A), PKC (protein kinase C) and CaMKII ( $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II). These kinases, as well as the tyrosine kinase Src will participate in the phosphorylation of NMDA and AMPA receptors, resulting in increased sensitivity due, for example, to the operation of NMDA receptors at resting membrane potential. PKC will also increase the trafficking of AMPA receptors to the cell membrane. Downstream of PKA and PKC, ERK (extracellular-signal-regulated kinase, a mitogen-activated protein kinase or MAPK) can also activate the potassium channel  $\text{K}_{v4.2}$ .

This MAPK is involved in the maintenance of central sensitisation: ERK can indeed be translocated to the nucleus and activate some transcription factors such as CREB (cAMP-response-element-binding protein) and Elk-1, which will bind to cAMP response elements (CRE) or serum-response elements (SRE) on gene promoter regions respectively. Consequently, the transcription of immediate early genes (IEG) and late-response genes (LRG) will then commence.

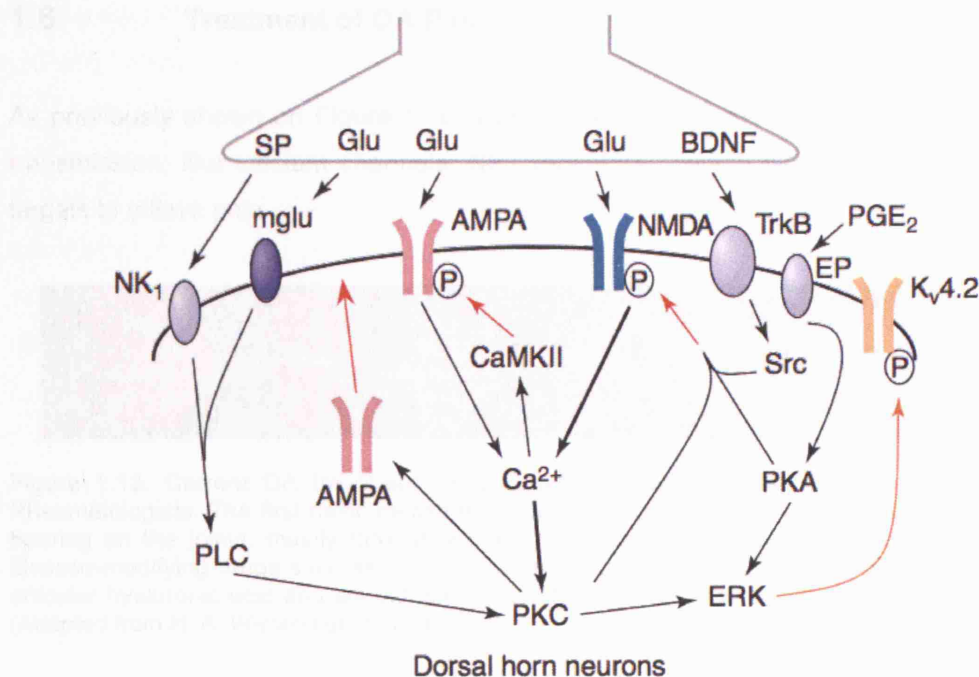


Figure 1.12. Induction of central sensitisation in dorsal horn neurones (R. R. Ji et al., 2003).

These two phenomena, **peripheral and central sensitisation**, play an important role in various chronic pain conditions, from the onset to the maintenance.

They can explain **allodynia**, i.e. pain due to a stimulus that does not normally provoke pain, as well as primary and secondary **hyperalgesia**, i.e. an increased response to a stimulus that is normally painful.



## 1.6 Treatment of OA Pain

As previously shown on Figure 1.10, various ligands and receptors are involved in pain transmission, like calcium channels, NK1 receptors... which can then be used as drug targets to relieve pain.



Figure 1.13. Current OA treatments issued in the guidelines from the American College of Rheumatologists. The first basic measure is not pharmacological and consists in reducing weight bearing on the joints, mainly through weight loss and increased exercise. Then come the non disease-modifying drugs such as acetaminophen (paracetamol), COX-2 inhibitors, NSAIDs, intra-articular hyaluronic acid and steroid injections. The last measure is the joint replacement surgery (Adapted from H. A. Wieland et al., 2005).

The current recommended treatment options include both non-pharmacological and pharmacological measures (Figure 1.13). While the former ones consist of decreasing weight-bearing on the joint - and thus on cartilage - mainly through weight loss and increased physical activity, the latter can only aim at decreasing pain as no recognised disease-modifying drug – i.e. that would act on cartilage repair for example – is available.

As noted earlier, although inflammation may not be present all the time in the OA condition, it plays an important role. The production of inflammatory mediators such as prostaglandins derived from arachidonic acid by cyclo-oxygenase 1 (COX-1, constitutively expressed in the gastrointestinal tract e.g.) and 2 (COX-2, inducible after tissue damage e.g.) can be responsible for the pain produced. Consequently, NSAIDs (non-steroidal anti-inflammatory drugs) and COX-2 selective inhibitors in particular, along with paracetamol, are used as a first line treatment to fight pain. However, while paracetamol is not very potent in OA conditions, NSAIDs have side-effects – on the gastro-intestinal tract to different extents - that prevent their long-term use. Moreover, the recent withdrawal of rofecoxib (Vioxx<sup>TM</sup>), a selective COX-2 inhibitor, by Merck because of increased risk of thrombotic cardiovascular events, still challenges the clinician to evaluate the cost-benefit of the treatment in each patient. NSAIDs have been tested in the MIA-induced model of OA pain: acute diclofenac only produced a decrease in mechanical hyperalgesia on day 14 but not day 28, and had no effect on tactile allodynia (Fernighough et al., 2004) whereas only chronic celecoxib had a positive effect on hindpaws weight-bearing (Pomonis et al., 2005).

Concerning intra-articular drug delivery, there is no clear evidence for hyaluronic acid efficacy, while steroids, although they can provide an immediate pain relief, can have deleterious effects on cartilage and joints. They have not been used in our model yet.

Other treatments not shown on the diagram are also recommended by the American College of Rheumatology. These include topical applications of analgesics such as NSAIDs and capsaicin cream – aimed at desensitising vanilloid receptors - that are often used as mono- or complementary therapies in the treatment of hand and knee OA (C. L. Deal et al., 1991). The use of opioid receptor agonists such as morphine is often restricted to cases of intense pain that would not be responsive to any of the above treatments, and mainly in a very controlled hospital setting, as it is the case for terminal cancer patients undergoing paroxysmal pain episodes (Figure 1.14).

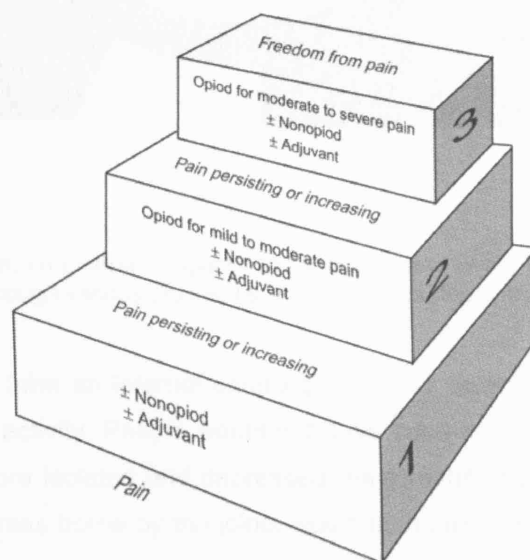


Figure 1.14. The WHO analgesic ladder. In order to reach a pain-free state in cancer patients, this ladder was proposed as a guideline for clinicians. Non-opioids drugs such as paracetamol or NSAIDs that can be complemented by adjuvants to circumvent the side-effects, are given as a first-line treatment. In case of pain persistence, NSAIDs can be temporarily co-administered with a mild opioid such as codein. Finally, step 3 involves the administration of a strong opioid such as morphine.

Thus, there is a situation whereby pain is difficult to treat, which can accelerate and worsen OA progression. A vicious circle is installed and needs to be broken.

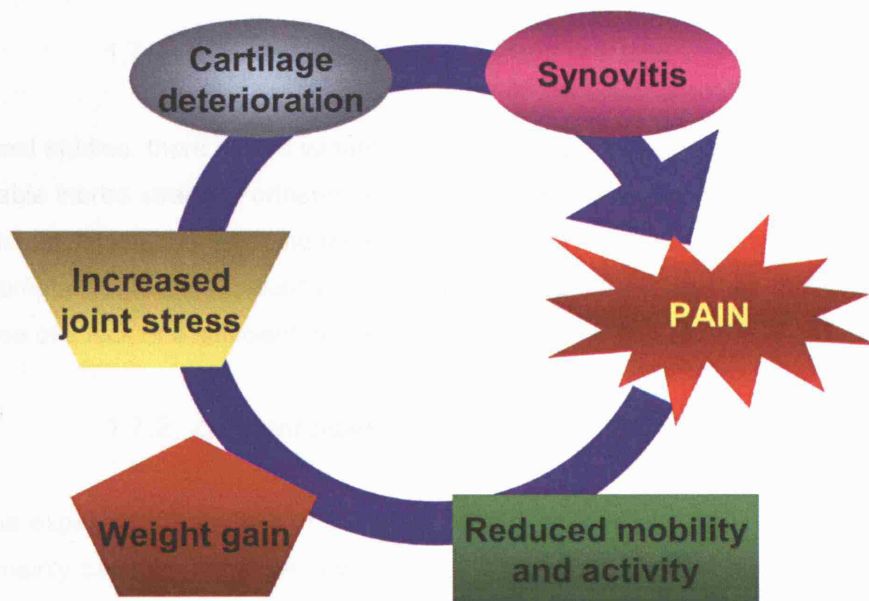


Figure 1.15. OA and Pain: an infernal coupling. The vicious circle where pain actually increases the strain put on the joints through various processes, which exacerbates further that pain.

Indeed, OA and pain form an infernal coupling. First, for example, joint pain can cause reduced mobility and activity. People would not have such an active social life as before and could become more isolated and depressed. As a result, they could gain weight and become obese: the stress borne by the joints would then increase, which would contribute further to cartilage degeneration and involve some episodes of synovitis causing pain again.

This vicious circle needs to be broken. The mechanisms of OA pain have to be unravelled, along with the development of new treatments. Animal models are very useful for that matter.



## 1.7 Preclinical models of OA

### 1.7.1 Why use animal models?

In animal studies, there is less variation between individual subjects as the rodents we use are stable inbred strains. Furthermore, using reliable preclinical models can be very useful for mechanistic studies as some techniques such as *in vivo* electrophysiology, immunology for example would be impossible to conduct in patients, for obvious ethical reasons and because of a lack of a sufficient number of patients.

### 1.7.2 Different types of models of OA

Various experimental models of OA have been described and have focused on the knee joint, mainly because knee OA is very frequent in human patients as well in large animals such as some breeds of dogs or horses.

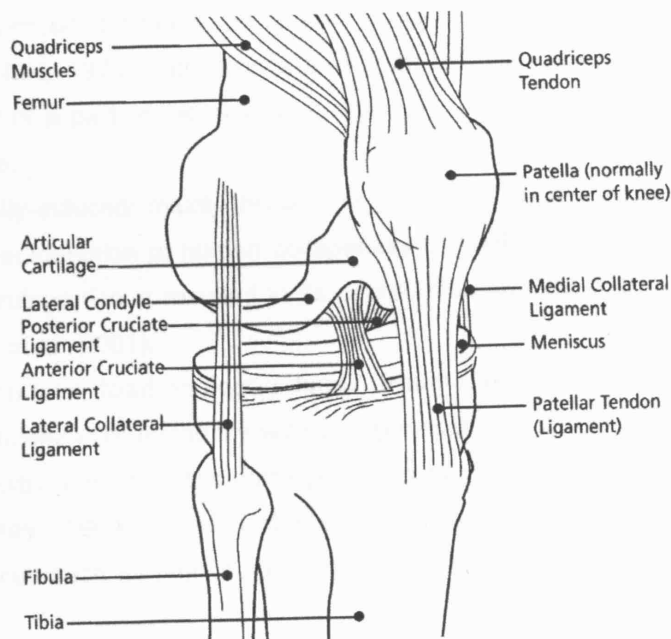


Figure 1.16. Anatomy of the knee joint: stability elements. The important elements that are of interest to us are the articular cartilage that is at the interface between the contact zones of the two bones (femur and tibia), with the lateral and medial menisci in between for a better stabilisation. Ligaments that are more likely to be torn and induce joint instability include the anterior cruciate ligament (ACL). Another useful anatomical structure for knee injections in the rat is the patellar tendon, which normally attaches the tibia to the quadriceps muscle, including the patella on its way. PCL: posterior cruciate ligament.

One big issue with OA is the fact that OA is a very heterogenic condition: is OA due to a knee instability that was once caused by a trauma e.g. a torn anterior cruciate ligament during a football match, to obesity, to normal ageing cartilage wear-and-tear, to a history of OA in the family, or to a combination of several factors. These are examples of how diverse OA conditions can be. It is thus important to bear in mind this heterogeneity. As a result, it is also important to use an animal model as close as possible to the human condition to be mimicked.

Generally speaking, OA can be induced by any form of joint instability or joint laxity then followed by altered weight-bearing on cartilage. The various existing models of OA can be classified upon the aetiology of OA. There are:

- naturally occurring models of OA: a reliable spontaneous OA can occur in some animal species but not in others, especially in the knee joints. Examples include mice, guinea pigs (OA starting at around 3 months) and Syrian hamsters;
- surgically-induced models: by anterior cruciate ligament transection (M. J. Pond and G. Nuki, 1973) mimicking a torn ligament, by partial medial meniscectomy (removal of a part of the meniscus cartilage), or by destabilisation of the medial meniscus;
- genetically-induced: mainly through the use of transgenic mice. For example, the post-natal induction of human collagenase-3 (MMP-13) protein overexpression in mice cartilage tissue resulted in its degradation and OA pathological signs (L. A. Neuhold et al., 2001);
- other models: load impact-induced trauma (B. Mazieres et al., 1987), or enzymatically joint instability with collagenases e.g. (P. M. van der Kraan et al., 1990), extracellular matrix damages using papain, a protein-cleaving enzyme (D. G. Murray, 1964), or disturbances of chondrocyte metabolism induced by substances such as monosodium iodoacetate (MIA) (D. A. Kalbhen and U. Blum, 1977).

### **1.7.3 The MIA-induced model of OA pain**

#### **1.7.3.1 History**

The MIA-induced model of knee osteoarthritis was first described in hens by Kalbhen and Blum in 1977. Ten years later, Kalbhen (1987) characterised the model in rats along with some pharmacological experiments.

The histopathology first showed that at late stages, subchondral bone was exposed, synovium damaged and joint pain similar to the human OA condition (C. Guingamp et al., 1997; R. E. Guzman et al., 2003). Then it was shown that the histopathology of the degenerating joint, at different time points, also resembled that seen in humans (Janusz et al., 2001). Inflammation was found to be resolved after day 7 within the synovium and surrounding tissues (S. E. Bove et al., 2003). The model also showed a very good correlation between histological findings and behaviour, suggesting its usefulness in studying OA (K. Kobayashi et al., 2003). Since then, different groups have used the same model (same dose of 2 mg MIA per animal into the knee joint) of OA: Combe et al. (2004) qualified it as a “model of chronic nociceptive pain in rats” because of the reliable chronic pain state after only 14 days, as shown in Figure 1.17. Fernighough et al. (2004) also characterised the model behaviourally and histologically and performed pharmacological studies comparing the MIA model with another model of OA based on partial medial meniscectomy: they showed an increased robustness in the MIA model in terms of behaviour supporting the reliability and usefulness of that model.

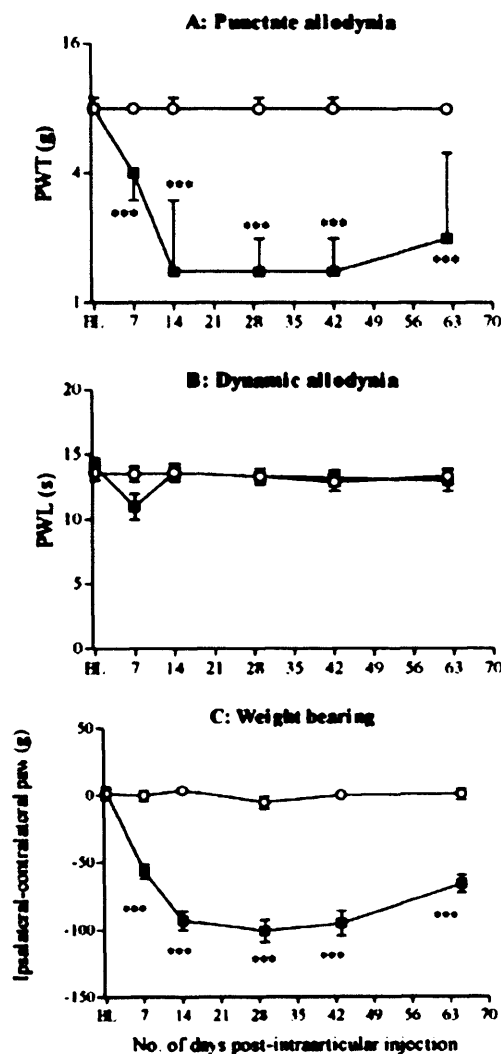


Figure 1.17. Development of punctate (A) and dynamic (B) allodynia, and weight bearing deficit (C) following intraarticular injection of MIA or saline. With all the parameters measured, a persistent pain behaviour was established from day 14. A. PWT (paw withdrawal thresholds) to von Frey hairs. B. Paw withdrawal latencies (PWL) to cotton bud. C. Hindpaw weight distribution (From R. Combe et al., 2004).

More recently, Pomonis et al. (2005) studied a similar MIA model of OA, comparing it to papain intra-articular injection and showed behavioural, radiographic and histological changes in agreement with previous findings for the MIA model. They also concluded that whereas MIA injection induced chronic joint degeneration, papain injection did not, which supported the relevance of the former model for the study of OA pain. Pain behaviour as well as bone destruction and cartilage damage in two arthritic models in guinea pig and were also characterised using fMRI (H. Vermeirsch et al., 2007). The MIA model was also used to test the effects of lacosamide, a member of a family of functionalised amino acids

(analogues of endogenous amino acids and D-serine): a reduction of in several pain modalities was observed (B. Beyreuther et al., 2007).

### 1.7.3.2 Mechanisms of action: MIA, an inhibitor of glycolysis

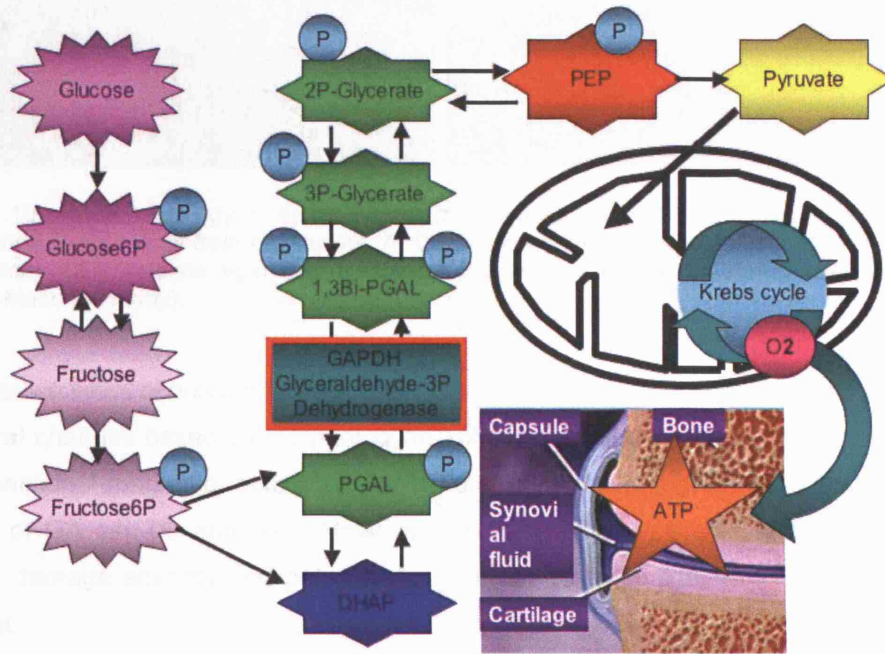


Figure 1.18. Glycolysis pathways and site of action of monosodium iodoacetate (MIA). MIA is an inhibitor of the glyceraldehyde-3 phosphate dehydrogenase (GAPDH): the usual Krebs' cycle occurring in mitochondria and leading to the production of ATP through the respiration phenomenon is thus inhibited, leading to an impaired chondrocyte metabolism and progressive cartilage damage.

MIA, an inhibitor of glycolysis often used in enzymatic studies, inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity because of the reaction of the iodoacetate with the cystein residue position 149 in particular (E. Recker, 1961; M. I. Sabri and S. Ochs, 1971). As a result, as shown on Figure 1.18, glucose cannot undergo the glycolysis cascade. The production of pyruvate and thus the Krebs cycle that normally occurs inside mitochondria to produce ATP (Adenosine Tri-Phosphate) are inhibited. As ATP is the basic cellular energy molecule, cell metabolism is inhibited and may result in death. The cells of interest to us are chondrocytes, responsible for cartilage production. They have a low basal metabolism and absorb all their nutrients from the synovial fluid. By injecting MIA directly into the synovial capsule, the drug will remain in direct contact with chondrocytes and alter their activity, resulting in cartilage degradation.

## 1.7.3.3 Outcome measures

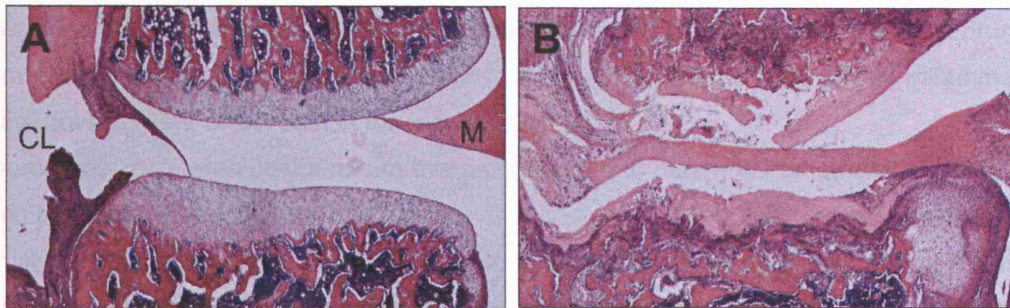


Figure 1.19. Knee histology (Hematoxylin-eosin staining) day 17, showing advanced joint degeneration in an animal from my studies. A. Control knee, medial condyle, B. MIA-injected knee. M. Meniscus CL: Cruciate ligament. Histology courtesy of Dr. H. Guehring (Sanofi-Aventis, Frankfurt-Main, Germany).

There are two kinds of outcome measures in OA.

**Structural** changes based on morphology/morphometry using imaging techniques such as X-ray, functional magnetic resonance (fMRI), or computed tomography (CT). Quantitative features of OA can be analysed. However, because of the variable level of correlation between damage severity and patients' pain score, other qualitative measurements are important.

**Functional** – more qualitative - measurements are based on different questionnaires in humans like the WOMAC or Western Ontario and McMaster Universities Arthritis Index, a questionnaire used to assess the symptoms of pain, stiffness and physical function in knee and hip osteoarthritis using 24 questions (N. Bellamy et al., 1988), and on a battery of tests in animals: mechanical, cooling testing, ambulation, which will be used in the experiments carried out by myself. As animals are not able to tell us what their pain is like, there is a need for techniques in order to assess their pain indirectly.

Intuitively, it can be expected that the nature of the pain tested in the hind paw, area of secondary hyperalgesia, will be different from the one tested using with the rotarod or the incapacitance tester for example, which depend more on the knee joint, where primary hyperalgesia develops. Those different tests can then provide us with useful and complementary information in our attempt to investigate the development of this chronic pain state.

## 1.8 Understanding the mechanisms of OA pain

How does OA pain arise? After assessing the effects of commonly used analgesic drugs in humans such as morphine and gabapentin, I wanted to investigate the role of inflammation in the development of OA pain in the model.

Neuropathy could also play a role in this pain model and hence immunohistochemistry was used to assess that component.

Finally, at the spinal cord level, *in vivo* electrophysiology coupled with the ablation of superficial NK-1 receptor expressing lamina I neurones was a powerful tool to understand neuronal mechanisms.

### 1.8.1 Morphine, gabapentin and OA pain

As shown in Figure 1.10, targets such as the opioid receptors and calcium channels can be used for pain relief, due to their distribution all over the nervous system.

Similar to cancer pain, the WHO ladder for pain relief (Figure 1.14) involves opioids in patients with severe pain when the usual NSAIDs are ineffective or have reached their maximal dosing regimen because of their side-effects.

#### 1.8.1.1 Morphine

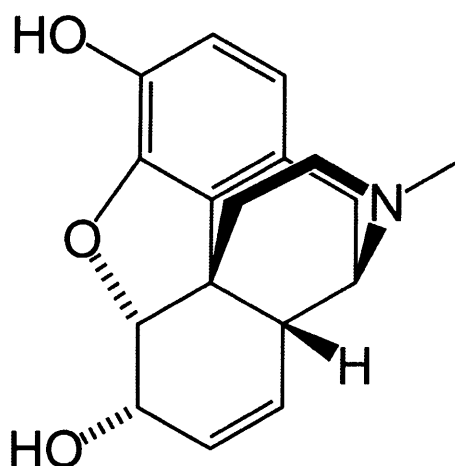


Figure 1.20. Structure of morphine.

The effects of poppy plants, producing opium, have been reported as early as 5.000 BC, and it is only in the early 1800s that Serturner isolated morphine as the active ingredient of opium, and as a member of the opioid family. Naloxone, a morphine derivative, was also found to be an antagonist of morphine activity. Three opioid receptor subtypes were traditionally distinguished from their pharmacological properties: mu (mainly responsible for morphine effects), delta and kappa. However, the molecular characterisation of these receptors only occurred in the 1990s and led to the discovery of an opioid receptor gene family, including the closely related ORL1 (opioid receptor-like 1) receptor, whose endogenous ligand is nociceptin, but whose opioid activity is discussed (F. Taylor and A. Dickenson, 1998). Opioid receptors are G-protein coupled receptors (GPCRs) and thus have a typical seven hydrophobic transmembrane domain structure; they are believed to couple to  $K^+$  and  $Ca^{2+}$  channels, resulting in analgesia. As shown on Figure 1.10.B, opioid receptors are present pre- and post-synaptically, suggesting different mechanisms of action. The first effect is caused by pre-synaptic opioid receptors that will, upon activation, cause the opening of  $K^+$  channels e.g., leading to the inhibition of neurotransmitter release in the synaptic cleft; the second effect will be the inhibition of neuronal firing when the receptor is found post-synaptically. The latter effect is dual: as no action potential is produced, less input is received by higher centres i.e. the brain. However, there is also a large population of inhibitory GABAergic interneurons. If those interneurons stop receiving input, the disinhibition that normally takes place is levied, potentially leading to a downstream activation of other neuronal populations. These effects occur at different levels in the nervous system, the most important effects being in the spinal cord, in the midbrain and the brainstem. The various opioid receptor subtypes have different distribution patterns throughout the nervous system (D. Besse et al., 1990) and each subtype was shown through the use of transgenic knockout mice to have very specific antinociceptive characteristics (M. Martin et al., 2003).

Opioids such as tramadol – that also inhibits reuptake of norepinephrine and serotonin – are used in some conditions like OA. Morphine proved to be efficient in OA patients; however, because of the possible side effects such as constipation and nausea, its use is rather reserved to moderate-to-severe OA pain (J. R. Caldwell et al., 2002). A review by Kalso et al. (1997) also provided some support for the analgesic efficacy of intra-articular morphine after knee surgery, although convincing evidence is rather lacking. In the MIA model of OA pain, different groups have recently tested acute morphine effects at different time points and showed positive results. Fernighough et al. (2004) showed a significant effect on the reduction of both mechanical hyperalgesia and tactile allodynia at days 14



and 28 post-induction. Combe et al. (2004) also found a reduction of punctuate allodynia and a change in hindpaws weight distribution.

### 1.8.1.2 Gabapentin

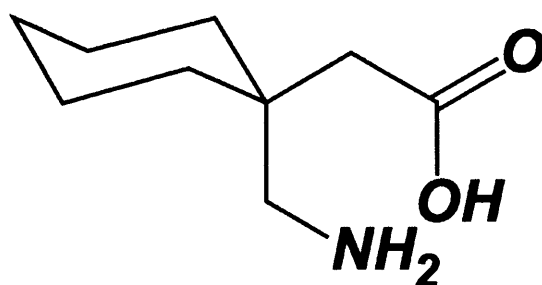


Figure 1.21. Structure of gabapentin.

Calcium channels also play an important role in pain transmission. The former antiepileptic drug gabapentin (1-(aminomethyl)cyclohexaneacetic acid, Neurontin<sup>TM</sup>, a structural analogue of GABA) was shown to decrease SP and CGRP release from rat spinal tissues after inflammation (J. C. Fehrenbacher et al., 2003). Gabapentin is likely to inhibit neuronal voltage-dependent calcium channel activity via an auxiliary subunit of the alpha-2-delta family (M. J. Field et al., 2000), resulting in decreased neurotransmitter release and action potential propagation. Being the only drug licensed for the treatment of neuropathic pain in the UK, gabapentin and now pregabalin (Lyrica<sup>TM</sup>), a derivative of gabapentin, are tested in various other chronic pain conditions and animal models. No clinical trials have investigated the effect of gabapentin on the treatment of OA in patients. In the MIA model of OA, gabapentin has been used acutely with an effect on tactile allodynia on day 14, but not on day 28, and no effect on mechanical hyperalgesia at any time-point (J. Fernihough et al., 2004).

Gabapentin obeys first-order elimination kinetics *via* urine. Less than 2% is metabolised in rats, its binding to proteins is minimal (<3%), and it does not induce hepatic P450. Following a single 50 mg/kg oral or intravenous dose, mean elimination half-life was 1.7±0.6 and 1.7±0.3 hour respectively in rats and repeated administration did not alter gabapentin pharmacokinetics. The plasmatic peak was also found at 1.7±0.8 hour following oral administration (L. L. Radulovic et al., 1995).

### 1.8.2 Inflammation in OA pain

Osteoarthritis was long believed to be a degenerative disease only, with no inflammatory component, hence the name “osteoarthrosis” used for some time.

Those two statements are now thought to be untrue, as:

- there is a bone-producing process accompanying bone destruction in OA, leading to the formation of osteophytes,
- inflammation, even if not present all the time, could appear in peaks, and thus trigger pain, for example after the release of osteocartilaginous microfragments following a long period of exercise.

I wanted to investigate the role of inflammation in this model of OA.

#### 1.8.2.1 The inflammatory cascade

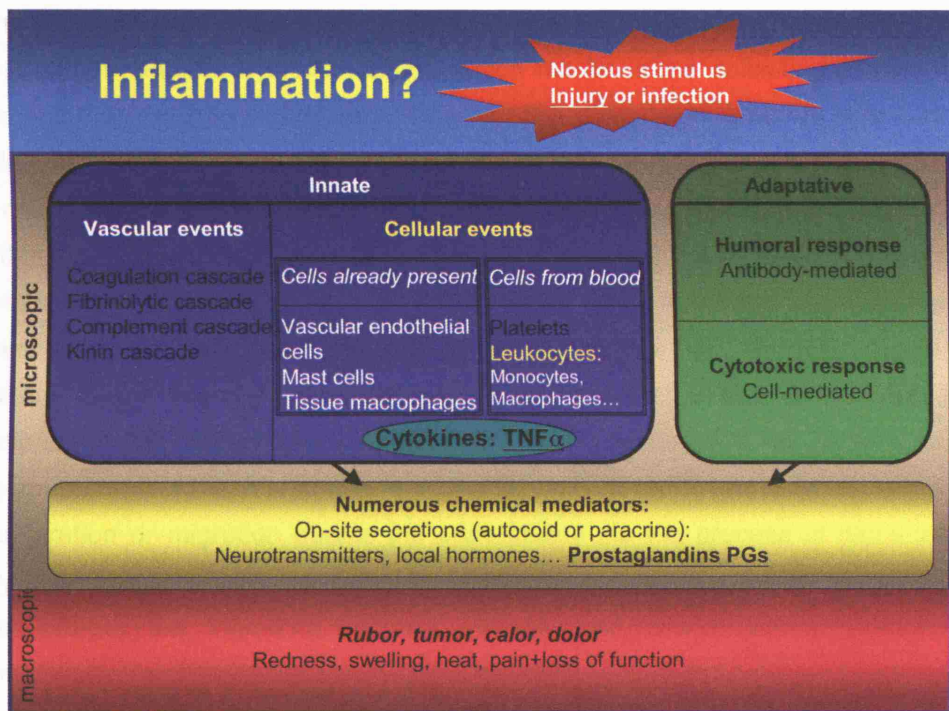


Figure 1.22. The inflammatory cascade, from the injury to the release of inflammatory mediators.

The typical inflammatory response can be induced by a noxious stimulus, an injury, an infectious agent (bacteria for example), or a combination of those. It will involve the four well-known macroscopic, classical signs of inflammation *Calor, dolor, rubor, and tumor*, or

redness, swelling, heat and pain, respectively, which were originally recorded by the Roman encyclopaedist Celsus in the 1<sup>st</sup> century AD.

Underlying those visible signs are some microscopic events due to reactions of the immune system. Indeed, following those stimuli, an immediate, innate immune response will occur, as well as an adaptative response, mainly in the presence of an infectious agent. OA pain is different from pain due to a septic arthritis (involving an infection of the knee joint due to some bacteria for example), so we can assume that the adaptative response will be minor in comparison to the innate response that we are going to study in detail.

In the innate response, two kinds of events can be distinguished.

The vascular events, with an exudative component, involve the movement of plasma fluid, containing important proteins such as fibrin and immunoglobulins (antibodies), into inflamed tissue. This movement is achieved via the chemically-induced dilation and increased permeability of blood vessels, which results in the collection of fluid into the tissue and thus swelling and oedema. However this component is not as important as the cellular events.

The cellular component involves the activation of resident cells, which will lead to release of inflammatory mediators (such as bradykinin) followed by the production and release of pro-inflammatory cytokines by newly arrived macrophages and other immune or immune-related cells. In fact, white blood cells or leukocytes that normally reside in the bloodstream are recruited and directed to the inflamed tissue via extravasation to participate in the inflammatory response. These mechanisms will involve leukocyte margination and adhesion to the endothelial cells local to the site of inflammation, transmigration or migration across the endothelium via the process of diapedesis, and finally chemotaxis enabling movement of leukocytes within the tissue. Some leukocytes act as phagocytes, ingesting bacteria, viruses, and cellular debris. Others release enzymatic granules that damage pathogenic invaders.

Leukocytes through the release of inflammatory mediators that develop and maintain the inflammatory response are critically involved in the initiation and maintenance of inflammation.

During an inflammatory response, not only inflammatory factors such as lysosomal enzymes will be released (by granulocytes e.g.): several cell-derived chemical mediators

such as neurotransmitters, local hormones, cytokines (a group of proteins and peptides that are used in as signalling compounds such as interferon-gamma secreted by T cells and natural killer (NK) cells, TNF-alpha, or tumor necrosis factor-alpha, and IL-1 primarily released by macrophages) will contribute to the release and activation of other enzymes such as PLA-2 (phospholipase A2) or COX, which could lead to the increased production of eicosanoids, leukotrienes and prostaglandins for example (lipids responsible for vasodilation, fever, and pain). Those prostaglandins, such as PGE2, could in turn cause pain and hyperalgesia through sensitisation of nociceptors.

That is what I am going to explain thereafter.

### 1.8.2.2 Lipids and cell membranes

In order to explain how some drugs can fight inflammation, it is important to understand what is happening at the cell membrane level, where prostaglandins are mainly produced.

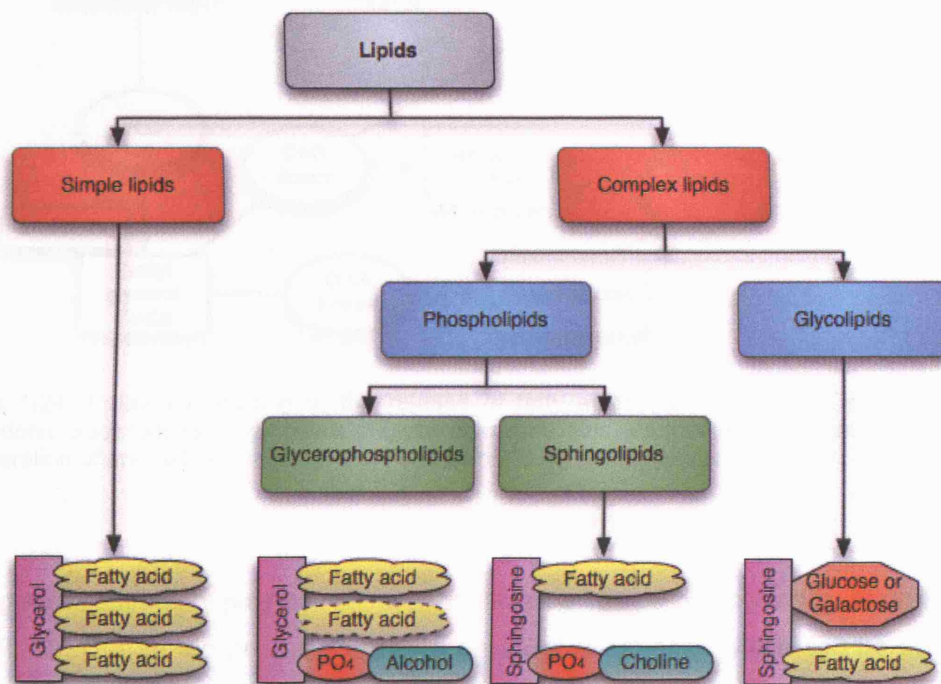


Figure 1.23. Schematic diagram of simple and complex lipids.

Cell membranes are mainly made of lipids organised in bi-layers, constituting a fluid mosaic. Lipids are water-insoluble substances that can be divided into four groups:

-**fats** (triglycerides made of fatty acids and glycerol) and waxes,

-**complex lipids**, important components of cell membranes. They can be divided into **phospholipids** (made of one central alcohol (glycerol or sphingosine), fatty acids like arachidonic acid and a nitrogen-containing phosphate ester, such as phosphorylcholine or inositol phosphate) and **glycolipids** containing sphingosine and a fatty acid associated with a carbohydrate portion such as glucose or galactose,

-**steroids**, the most common being cholesterol, an important precursor for the synthesis of other steroids such as bile salts and hormones (sexual and adrenocorticoid for example),

-**eicosanoids**: prostaglandins, thromboxanes and leukotrienes, mainly derivatives of the polyunsaturated fatty acid arachidonic acid, and involved in blood pressure regulation, anaphylactic shock, fever, blood clotting, inflammation... In general, eicosanoids are only produced in response to a stimulation (cellular lesion, release of an inflammatory mediator) and their degradation is very rapid.

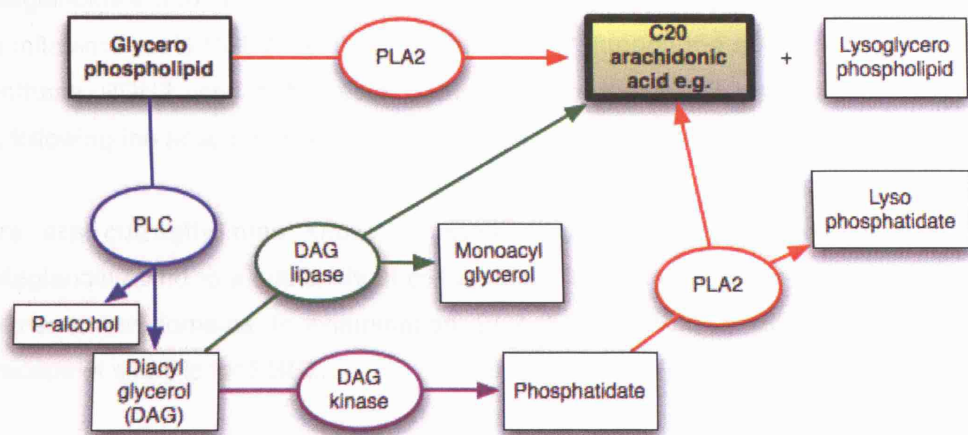


Figure 1.24. Pathways leading to the release of fatty acids (containing 20 Carbons such as arachidonic acid) following hydrolysis of glycerophospholipids. Various enzymes can be involved in the liberation of arachidonic acid, the main one being PLA2 (phospholipase A2).

During an inflammatory process, some phospholipids can be hydrolysed by phospholipase A2 (PLA2). One of the products will then be arachidonic acid, which can be used as a substrate and transformed through the action of various enzymes such as lipoxygenases, epoxigenases and cyclo-oxygenases (COX), into eicosanoids.



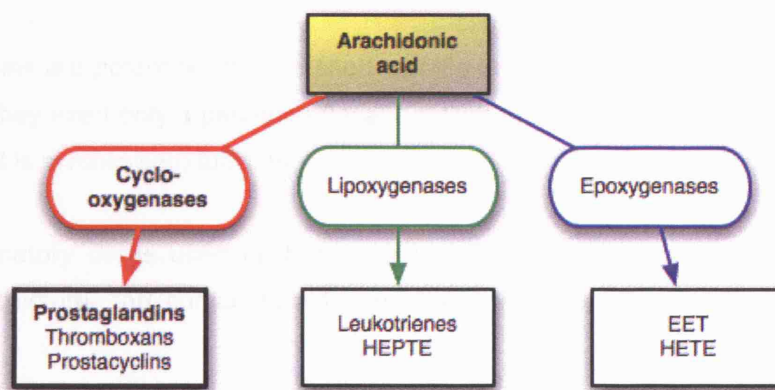


Figure 1.25. Production of eicosanoids following the release of arachidonic acid, through various enzymes. In pain and inflammation, it is mainly the cyclo-oxygenase (COX) enzymes that are relevant as they participate in the production of prostaglandins.

Prostaglandins are found in virtually all tissues and organs. The prostaglandin of interest to us in inflammation is PGE<sub>2</sub>, synthesised from PGH<sub>2</sub> through the action of a prostaglandin E synthase. PGH<sub>2</sub> itself is the result of the action of the PGH<sub>2</sub> synthase on arachidonic acid, following the action of PLA<sub>2</sub> on phospholipids.

There are currently nine known prostaglandin receptors on various cell types. Prostaglandins bind to a subfamily of cell surface G-protein-coupled receptors with seven-transmembrane domains. In inflammation, EP<sub>2</sub> (E prostanoid 2) receptors correspond to the receptors specific for PGE<sub>2</sub>.

Due to the variety of existing receptors, prostaglandins can act on a variety of cells, and have a wide variety of actions. For example, they can cause constriction or dilatation in vascular smooth muscle cells, cause aggregation or disaggregation of platelets, constrict smooth muscle, regulate calcium movement, regulate hormone regulation, control cell growth and of course sensitise spinal neurones to nociceptive input and regulate inflammatory mediation, especially for PGE<sub>2</sub>.

Indeed, prostaglandins, in particular PGE<sub>2</sub>, can activate the G<sub>s</sub>-coupled PGE<sub>2</sub> receptors, which causes the activation of adenylate cyclase, a secondary messenger, by the alpha subunit of the heterotrimeric G-protein associated with the receptor. Adenylate cyclase produces cAMP from ATP, which then in turn activates PKA (protein kinase A) inducing the phosphorylation of Na<sup>+</sup> ion channels, their opening and the generation of an action potential.

Prostaglandins are potent but have a short half-life before being inactivated and excreted. Therefore, they exert only a paracrine (locally active) or autocrine (acting on the same cell from which it is synthesised) function.

Anti-inflammatory drugs used in therapy are divided into two groups, based on their chemical structure: the corticosteroids and the non-steroidal anti-inflammatory drugs (NSAIDs).

### 1.8.2.3 Steroids and mode of action

Natural corticosteroids are steroid hormones produced by the adrenal glands: 95% of the secretions mainly have a glucocorticoid action (they can increase glycaemia - blood glucose concentration), whilst 5% have a mineralocorticoid action (they act on blood pressure and water-sodium retention). Cortisol (hydrocortisone) and aldosterone, natural glucocorticoid and mineralocorticoid hormones respectively, are important adrenocorticoid hormones derived from progesterone.

A variety of synthetic glucocorticoids, some far more potent than cortisol, the standard of comparison for glucocorticoid potency, have been created for therapeutic use.

<b>Drug</b>	<b>Glucocorticoid effect</b>	<b>Mineralocorticoid effect</b>	<b>Duration of action (<math>t_{1/2}</math> in hours)</b>
<b>Hydrocortisone (cortisol)</b>	1	1	8
<b>Prednisolone</b>	3.5-5	0.8	16-36
<b>Methylprednisolone</b>	5-7.5	0.5	18-40
<b>Dexamethasone</b>	25-80	0	36-54
<b>Betamethasone</b>	25-80	0	36-54
<b>Triamcinolone</b>	5	0	12-36
<b>Aldosterone</b>	0.3	200-1000	-

Table 1.6. Comparison of different steroids' properties following oral administration (Pharmacology, G. M. Brenner, 2000).

Table 1.6 shows a few examples of steroids available and used in the clinics. They have different properties in terms of both their glucocorticoid and mineralocorticoid effects, and in terms of duration of action.



## Mechanism of action of glucocorticoids

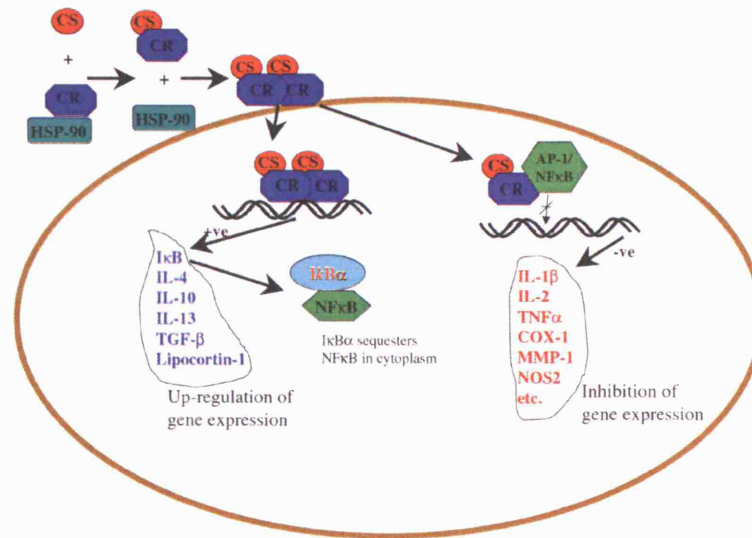


Figure 1.26 Following their internalisation into the cytoplasm, corticosteroids (CS) bind to their receptor to form the corticosteroid/receptor complex (CS/CR). Receptor dissociates from the inactive, multi-protein complex. The CS/CR complex translocates to the nucleus to bind to the specific DNA motifs, the glucocorticosteroid response elements (GRE). GRE can mediate both positive and negative corticosteroid effects. It also interferes with the transcriptional activity of AP-1 and NF-kappa B. Corticosteroids upregulate I-kappa B production that binds to NF-kappa B and sequesters it in the cytoplasm by inhibiting its nuclear translocation. (From I. C. Chikanza and D. L. Kozaci, 2004).

Corticosteroids first enter cells where they bind to the cytosolic glucocorticosteroid receptors, in response to which a large number of intracellular events are entrained. The first event is transactivation, where the newly formed ligand-receptor activated complex enters the cell nucleus and binds to the DNA glucocorticoid response elements (GREs) in the promoter region of some target genes. The opposite mechanism, transrepression, involves the binding of ligand-receptor complex to non-DNA molecules such as specific transcription factors, which will prevent the transcription of targeted genes, such as immune genes coding for some interleukins.

Ordinarily, glucocorticosteroids will trigger both processes of transactivation and transrepression and can influence both immune genes responsible for the inflammatory response, but also other genes involved in metabolic and cardiovascular functions. The ideal glucocorticosteroid would only suppress the immune response.

In the specific case of an inflammatory response, glucocorticoids mainly act by inhibiting the activity of PLA2 whose activity is increased for example by cytokines following

stimulation, in our case an injury. This inhibition takes place through the two mechanisms detailed above:

- by repressing some immune system genes, glucocorticoids will directly inhibit the expression of COX-2, and thus the synthesis of some derivative products of arachidonic acid such as prostaglandins,
- by inducing the production of lipocortins in target cells: lipocortins are not direct inhibitors of PLA2; however, in the presence of  $\text{Ca}^{2+}$ , they will bind to phospholipids, hence blocking their hydrolysis by PLA2 and the subsequent release of arachidonic acid that would normally be followed by the formation of eicosanoids responsible for inflammation.

Glucocorticoids could also act on cell membranes to alter ion permeability as well as modify the production of neurohormones.

It is important to distinguish between the physiological effects (replacement therapy) and the pharmacological effects (occurring at higher doses) of glucocorticoids as their production is physiological. As a result, some side-effects are associated with long-term use of glucocorticoids and non-steroidal anti-inflammatory drugs acting on COX enzymes are available.

#### 1.8.2.4 NSAIDs

Non-steroidal anti-inflammatory drugs, or NSAIDs, are characterised by three pharmacodynamic properties: they are analgesic, antipyretic and anti-inflammatory.

##### **Brief history**

They were already used in Antiquity with the folk remedy willow (*Salix*) bark containing salicylic acid as found on an Egyptian papyrus dated from 1550 BC. Whilst in 1763 Reverend Stone reported the anti-rheumatic properties of willow bark, it is only in 1827 that Leroux first isolated its active compound, salicin, a compound associated with antipyretic effects. The products of the hydrolysis of salicin are glucose and salicylic alcohol. The latter can be transformed in salicylic acid. As a result of its bad taste, Hoffman, a chemist from Bayer laboratories, produced acetylsalicylic acid to treat his father suffering from rheumatism. Acetylsalicylic acid was then used as a drug under the name of aspirin in 1899 thanks to Dresser. Until the discovery of the anti-inflammatory

properties of corticosteroids in 1947 by Hench, acetylsalicylic acid was the only anti-inflammatory drug available.

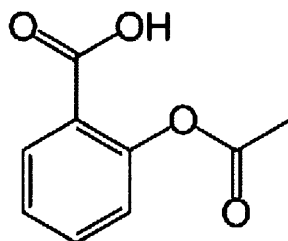


Figure 1.27. Structure of acetylsalicylic acid or aspirin.

Since then, other NSAIDs have been developed, most of them being organic acids.

### Mechanisms of action

This mechanism of action of NSAIDs was elucidated by John Vane in 1971 (J. R. Vane, 1971), who later received a Nobel Prize for his work. He showed the non-selective inhibition of the cyclo-oxygenase enzyme, including both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes, involved on the synthesis of eicosanoids (**prostaglandins**, thromboxane, prostacyclins) from arachidonic acid (Figure 1.25).

### COX enzymes

COX enzymes are expressed under at least two well-characterised isoforms: COX-1 and COX-2. COX-1 is constitutively expressed and observed under physiological conditions and is responsible for the synthesis of prostaglandins that protect the organism. COX-2 is the isoform induced by inflammatory stimuli and pathological conditions (J. L. Masferrer et al., 1992). NSAIDs are classified depending on the activity of each isoform as non-selective

Various COX inhibitors such as aspirin or ibuprofen are available. Acetaminophen (or paracetamol) is often classified under that heading although its mode of action is unclear. Newly developed selective COX-2 inhibitors are currently the subjects of increased attention.

Drug	Relative potency	Half-life (hours)	Daily doses
<b>Non-selective COX inhibitors</b>			
Acetaminophen (paracetamol)	1	3	4
Aspirin	1	2*	4
Ibuprofen	4	2	2-4
Indomethacin	40	4	1-3
Ketoprofen	20	2	2-4
Ketorolac	100	7	4
Naproxen	4	14	2
<b>Selective COX-2 inhibitors</b>			
Celecoxib	20	11	2

Table 1.7. Properties of non-steroidal anti-inflammatory drugs. \* For aspirin, the value shown is the half-life of the active metabolite, salicylic acid (Pharmacology, G. M. Brenner, 2000).

Indeed, by inhibiting constitutive prostaglandins such as PGE<sub>2</sub> and PGI<sub>2</sub> that stimulate the production of gastric mucus and inhibit HCl (hydrochloric acid) secretion, most NSAIDs will incur gastrointestinal damage in both humans and animals, and they are the first iatrogenic cause of mortality in patients. Indeed, they are associated with nausea, erosion and ulceration of the gastric or intestinal mucosa. Endogenous prostaglandins are also involved in the regulation of renal water excretion and blood flow, and their inhibition by NSAIDs can have serious consequences.

That is why so much emphasis has been put on the development of COX-2 specific drugs.

#### 1.8.2.5 Anti-TNF-alpha

Tumor necrosis factor-alpha (TNF-alpha, cachexin or cachectin) is a pleiotropic inflammatory cytokine mainly secreted by macrophages, and involved in the regulation of immune cells. It can cause various events such as apoptotic cell death, cellular proliferation, differentiation, tumorigenesis, viral replication and of course inflammation.

Dysregulation and, in particular, overproduction of TNF-alpha have been implicated in a variety of human diseases, including autoimmune diseases such as RA, insulin resistance, and cancer.

## Structure and receptors

TNF- $\alpha$  is a trimeric protein encoded within the major histocompatibility complex. It was first identified in its 17 kDa secreted form, but further research then showed that a non-cleaved 27 kDa precursor form also existed in transmembrane form (C. Perez et al., 1990). Stimulated macrophages produce 27 kDa TNF- $\alpha$ , which can bind directly to two receptors, TNF-R1 (TNF receptor type 1; CD120a; p55/60) and TNF-R2 (TNF receptor type 2; CD120b; p75/80). TNF-R1 is constitutively expressed in most tissues, and can be fully activated by both the membrane-bound and soluble trimeric forms of TNF, while TNF-R2 is only found in cells of the immune system and respond to the membrane-bound form of the TNF homotrimer. As most information regarding TNF signalling is derived from TNF-R1, the role of TNF-R2 is likely to be underestimated.

## Mechanisms of action

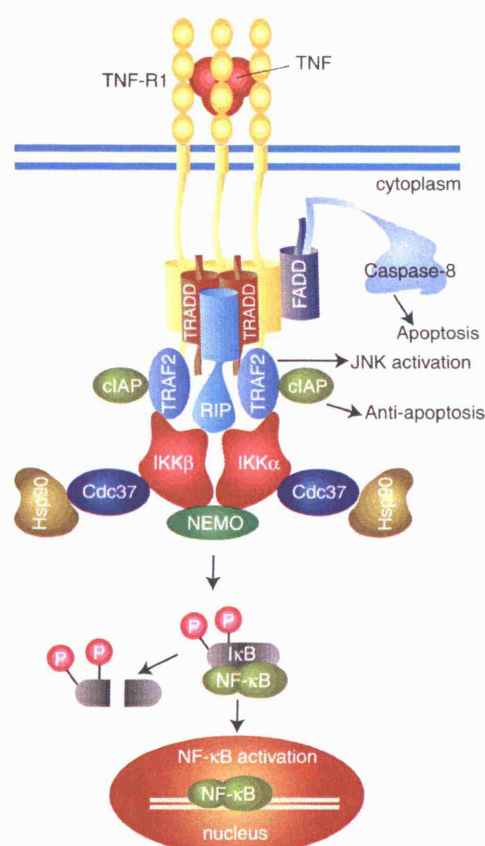


Figure 1.28. TNF signal transduction pathway. Engagement of TNF with its cognate receptor TNF-R1 results in the release of SODD and formation of a receptor-proximal complex containing the important adaptor proteins TRADD, TRAF2, RIP, and FADD. These adaptor proteins in turn recruit additional key pathway-specific enzymes (for example, caspase-8 and IKK-beta ) to the TNF-R1 complex, where they become activated and initiate downstream events leading to apoptosis, NF-kappa B activation, and JNK activation (From G. Chen and D. V. Goeddel, 2002).

Upon contact with their ligand, TNF receptors form trimers. This binding causes a conformational change in the receptor, leading to the dissociation of the inhibitory protein SODD from the intracellular death domain, which enables the adaptor protein TRADD to bind to the death domain, serving as a platform for subsequent protein binding. Following TRADD binding, three pathways can be initiated (G. Chen and D. V. Goeddel, 2002; U. Gaur and B. B. Aggarwal, 2003):

- Activation of NF-kappa B: TRADD recruits TRAF2 and RIP. TRAF2 in turn recruits the multicomponent protein kinase IKK, enabling the serine-threonine kinase RIP to activate it. Following the phosphorylation by IKK and consequent degradation of I-kappa B, an inhibitory protein that normally binds to NF-kappa B and inhibits its translocation, NF-kappa B is released. NF-kappa B is a heterodimeric transcription factor that can translocate to the nucleus and mediate the transcription of a vast array of proteins involved in cell survival and proliferation, **inflammatory response**, and anti-apoptotic factors.

- Activation of the MAPK pathways: of the three major MAPK cascades, TNF induces a strong activation of the stress-related JNK group, evokes moderate response of the p38-MAPK, and minimal activation of the classical ERKs. TRAF2 leads to JNK activation that translocates into the nucleus and activates transcription factors such as c-Jun and ATF2. The JNK pathway is involved in cell differentiation, proliferation, and is generally pro-apoptotic.

- Induction of death signalling: like all death-domain containing members of the TNFR superfamily, TNF-R1 is involved in death signalling. However, TNF-induced cell death plays only a minor role compared to its overwhelming functions in the inflammatory process. Its death inducing capability is weak compared to other family members (such as Fas), and often masked by the anti-apoptotic effects of NF-kappa B. Nevertheless, TRADD binds FADD, which then recruits the protease caspase-8. A high concentration of caspase-8 induces its autoproteolytic activation and subsequent cell apoptosis.

The myriad and often conflicting effects mediated by the above pathways indicate the existence of extensive cross-talk. For instance, NF-kappa B enhances the transcription of cFLIP, Bcl-2, and cIAP, inhibitory proteins that interfere with death signalling. On the other hand, activated caspases cleave several components of the NF-kappa B pathway, including RIP, IKK, and the subunits of NF-kappa B itself. Other factors, such as cell type,

concurrent stimulation of other cytokines, or the amount of reactive oxygen species (ROS) can shift the balance in favour of one pathway or another. Such complicated signalling ensures that whenever TNF is released, various cells with vastly diverse functions and conditions can all respond appropriately to inflammation.

### **Physiology**

Acutely, it was shown that TNF-alpha could indirectly sensitise nociceptive neurones via the induction of a proinflammatory cytokine cascade involving IL-1 beta, IL-6, and IL-8, which resulted in the release of prostaglandins and other mediators from immune cells (F. Q. Cunha et al., 1991; S. H. Ferreira et al., 1993; C. J. Woolf et al., 1997). Moreover, electrophysiological studies have provided more evidence of the direct actions of TNF-alpha as low-dose subcutaneous injections induced ectopic activity in nociceptive neurones within two minutes, and higher doses produced significant mechanical and thermal hyperalgesia by 15 minutes (L. S. Sorkin et al., 1997; H. Junger and L. S. Sorkin, 2000; A. Oprea and M. Kress, 2000). The application of TNF-alpha was also shown to enhance calcium currents and increase neuronal sensitivity to the neurotoxin capsaicin in cultures of sensory neurones (G. D. Nicol et al., 1997; N. Morioka et al., 2000; J. Pollock et al., 2002).

### **Pharmacology**

Tumor necrosis factor promotes the inflammatory response, which in turn causes many of the clinical problems associated with autoimmune disorders such as rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, psoriasis and refractory asthma. These disorders are sometimes treated by using a TNF inhibitor. This inhibition can be achieved with a monoclonal antibody such as infliximab (Remicade™) or adalimumab (Humira™), or with a circulating receptor fusion protein such as etanercept (Enbrel™).

### 1.8.3 *Neuropathy in OA pain*

Does OA pain have a neuropathic component? This is not really clear yet could have important consequences for OA treatment.

Moreover, in animals, it has been shown that knee denervation using an immunotoxin killed neurons after retrograde axonal transport and promoted OA in the aging rat (P. T. Salo et al., 2002), which shows the potential importance that neuropathy might play in OA development.

Contrary to inflammation that can be easily modulated with pharmacological agents, it is not currently possible to do the same with neuropathy. Indeed, some drugs such as gabapentin are indicated for the treatment of neuropathic pain. However, it was primarily developed to treat epilepsy; so this drug has other central effects that would not be easily separated from its effects on the neuropathy itself.

Another approach to assess the development of neuropathy is the use of immunohistochemistry.

ATF-3, a reliable and sensitive marker of neuronal damage expressed in DRGs, has been found to be expressed following axotomy in sensory and motor neurones. While DRG and spinal cord injury as well as formalin intraplantar injection induced the expression of ATF-3, intraplantar injection of Freund's adjuvant, producing mainly an inflammatory response, did not (H. Tsujino et al., 2000).

It was also necessary to identify the DRGs that were innervating the knee, as this was not very well characterised in rats. Previous studies using a retrograde labelling with the fluorescent dye Fluoro-Gold injected in the knee have shown that the L3 and L4 DRGs received most of the primary afferents input from the knee (P. T. Salo and E. Theriault, 1997).

Recently, an early neuropathic component was detected in this model, at the L5 DRG level only, but no later than day 14 post-induction (S. P. Ivanavicius et al., 2007).



#### 1.8.4 **Neuronal mechanisms**

The different neuronal populations in the dorsal horn of the spinal cord have been characterised in normal animals. Central sensitisation following OA induction could be investigated using *in vivo* electrophysiology by studying responses in arthritic rats. Indeed, following acute inflammation for example, spinal neurones show a gradual increase in their responses to innocuous and noxious pressure applied to the inflamed joint and surrounding, non-inflamed tissue (H. G. Schaible and B. D. Grubb, 1993). Some alterations in neuronal population characteristics, with increased responses of deep wide dynamic range neurones to electrical and thermal stimuli were demonstrated in a model of cancer-induced bone pain (C. E. Urch et al., 2003), but nothing has been done in models of OA.

Moreover, superficial NK-1 receptor expressing neurones were shown to be important in neuronal hyperexcitability and for descending facilitatory serotonergic controls from the brainstem onto the spinal cord in some preclinical models of pain (R. Suzuki et al., 2002). Again, nothing has been shown in this model of OA pain.

## 1.9 My PhD – Aims of this study

The first aim of my PhD was to validate the model using new behavioural techniques to characterise the model by studying responses to mechanical stimuli, cooling and movement.

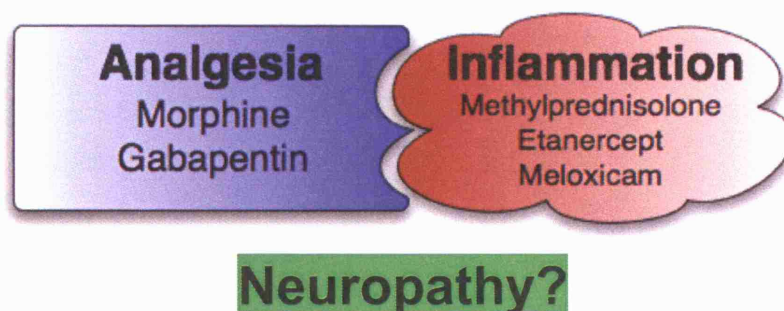


Figure 1.29. What I wanted to test in the model.

I wished to use this OA pain model to first study the effects of existing analgesic drugs such as morphine, as well as gabapentin, administered on a chronic dosing regimen to mimic human treatment conditions. The next step was to understand the role of inflammation during both the induction period, i.e. the first 14 days post MIA injection, and the subsequent chronic nociceptive pain state.

I also wanted to investigate the role of neuropathy in this model, as the contribution of neuropathy in the OA condition was not well known.

Understanding better this pain state through the neuronal changes and mechanisms was another part of the study.

Finally, I performed a final validation of the model by performing a fully characterisation of its responses to various doses of a novel peripheral opioid compound for a private pharmaceutical company, to show the relevance and usefulness of the model in testing new analgesic drugs.

## **Chapter 2. Methods**

## 2.1 Animals

Male Sprague-Dawley rats (Central Biological Services, University College London) weighing between 120 and 140 g at the time of training (cf. *infra*) were used. Sprague-Dawley rats are known to have a good temperament, a rapid growth and they are very good breeders. They were housed at a maximum of five per cage on a 12h day-12h night cycle with food and water available *ad libitum*. All experimental procedures were approved by the UK Home Office and follow the guidelines of the International Association for the Study of Pain (M. Zimmermann, 1983).

The rats were individually identified by indelible numbers inscribed on their tails.

Their weight was also recorded on each experimental day.

## 2.2 Induction of osteoarthritis pain

After clipping the hair covering the right knee, rats were anaesthetised by inhalation of a halothane (4% induction / 2% maintenance) in N<sub>2</sub>O/O<sub>2</sub> mixture (ratio 2:1) and placed on a heating blanket in the supine position (dorsal decubitus). The ventral surface of the right knee was then wiped with a chlorhexidine-based solution. 2 mg of monosodium iodoacetate (MIA, Sigma) in 25 µl of sterile saline (NaCl) was then injected as a slow bolus using a 27 G needle through the infrapatellar ligament of the right knee. Sham animals received saline only. Animals were closely monitored until anaesthesia recovery.

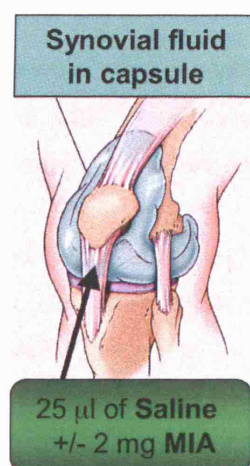


Figure 2.1. Site of injection for the intra-articular compounds. The patellar tendon attaches the quadriceps muscle to the tibia, with the patella included in it. The lower part of that tendon, between the tibia and the patella, is also called (infra)patellar ligament as it is joining two bones together. Adapted from a drawing by Marcia Hartsock, 2002.

For further reference in this report, the right, lesioned hindpaw will be termed ipsilateral (IL) and the left, non-lesioned paw, contralateral (CL).

The day of the MIA injection will be referred to as Day 0.

## **2.3 Behavioural studies**

The behavioural changes were assessed at different time points following the injections, in a blinded and randomised manner. The different testing procedures were standardised in order to increase consistency and will be detailed below. A wide range of behavioural techniques was used to study the different modalities of pain involved in this model.

### **2.3.1 *Animal training***

4 days before the surgery (referred as day -4), animals were selected from the animal stock on both weight and rotarod aptitude criteria. The rotarod apparatus will be detailed in the behaviour section.

The aim of the training was to familiarise the rats with testing procedures, to ensure the absence of locomotive defects and to eliminate poor rotarod performers (i.e. <60 sec. endurance), followed by baseline behavioural testing whose methods are detailed below.

### **2.3.2 *Acclimatisation***

Before starting any testing, animals were always acclimatised to the behaviour room for a minimum of 30 minutes: once transported there, they would stay for 15 minutes in their housing cage and would then be transferred to the testing Perspex cages for another 15 minutes before any test would start.

After that acclimatisation period, and before doing any tests, animals were expected to be fully awake (they were gently stimulated from below if necessary) and standing on their four paws. They were not tested whilst grooming, moving or licking themselves. Stress-induced hiccup should not be present either.

### **2.3.3 *Mechanical hypersensitivity***

Mechanical hypersensitivity was assessed using von Frey filaments (Touch-test<sup>TM</sup>, North Coast Medical Inc., San Jose CA, USA) providing forces of 1, 6 and 8 g, respectively 9.8, 58.9 and 78.5 mN. Each von Frey was applied a total of 10 times over the plantar surface of hindpaws (toes and pads), on both the ipsilateral (IL) and contralateral (CL) side to the injected knee. Stimuli had a 2-second duration and each VF hair was separated by 5 min at least, in ascending force (1, 6 then 8 g). The von Frey hairs were tested between the acetone tests (cf. *infra*).

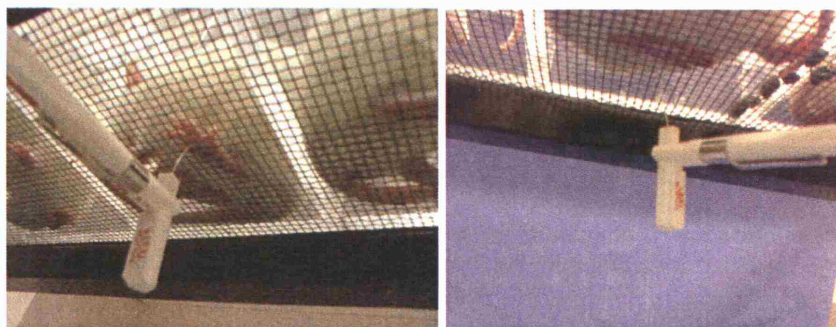


Figure 2.2. Von Frey filaments testing. The von Frey hairs of various bending forces are applied 10 times each through a mesh over the paw and toes and the responses are counted.

A test was considered positive in case of: consistent withdrawal or lifting of the whole paw (toes and pads) after bending of the VF hair, or paw biting behaviour. In case of doubt (surprise effect, VF hair slipping off the plantar surface or toes...), the test was repeated.

In case of guarding, each full paw (toe and heel) lifting due to the VF hair application was considered positive, whether the VF hair bent or not (no weight-bearing at all on hindpaw).

In all other cases, the test was deemed negative: for example if, when applying a VF hair to a toe, only the toe could be lifted up but not the heel.

Von Frey hairs were tested in ascending – and not in a random - order in order to avoid any kind of sensitisation, which could have slightly biased the responses recorded although this bias would be expected to be the same over all treatment groups due to the randomisation of the treatments.

#### 2.3.4 Cooling sensitivity

The cooling hypersensitivity was assessed using the acetone drop test: a drop of acetone was applied to both the ipsilateral then contralateral hindpaws using a 10 cm tubing connected to a 0.5 ml syringe.



The test was repeated for a total of 5 times with a minimum of 2 min between each application (VF tests would occur during that time). The presence or absence of a response was recorded and the response frequency (i.e. number of positive responses out of 5) was determined. Withdrawal responses and whole paw lifts were scored as positive.



Figure 2.3. The acetone drop test. The response frequency to 5 applications following the application of acetone on the plantar surface of the hindpaw were recorded.

Detailed criteria for the positive responses are given below.

<p><b>Positive:</b> delayed frank, marked, tapping, flinching, or shaking of large amplitude (could be quick), accompanied or not by toe/paw eating/biting.</p> <p><b>Negative:</b> early/immediate tapping of small amplitude to get rid of the liquid. Walking around the cage on tiptoes was also considered negative, unless shaking/lifting occurred.</p>
--

Table 2.1. Acetone test detailed scoring criteria.

2.3.5 *Ambulatory-evoked pain score, latency to fall*

An accelerating Rotarod device (Rotarod 7750, Ugo Basile, Italy) was used to assess ambulatory-evoked pain. The apparatus was set to accelerate from 0 to 20 revolutions per minute (rpm) in 60 seconds.

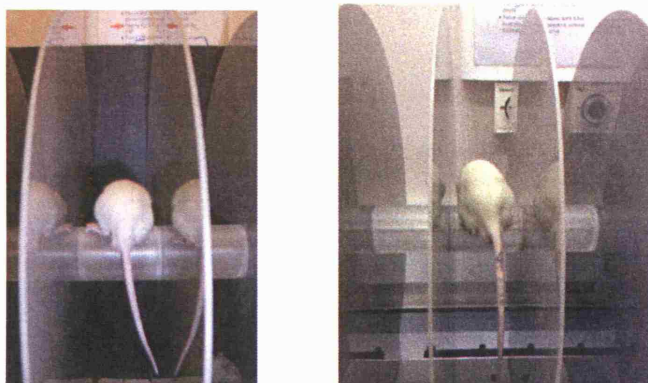


Figure 2.4. The Rotarod device. Rats were individually placed on a still rod before it started accelerating. The latency to fall and limping scores were then recorded.

The limp was then scored on a scale from 0 to 3 as described below, and the latency to fall off the rod, aiming to assess motor coordination/sedation, recorded with a maximum cut-off period of 180 seconds.

<b>0</b>	Absence of limp
<b>1</b>	Slight limp but no decreased usage of limb
<b>2</b>	Limp with decreased usage of lesioned limb
<b>3</b>	Total avoidance of usage of lesioned limb

Table 2.2. Ambulatory evoked pain score criteria.

Animals had to go through a training period, usually on day –4 or –3 before OA induction, where they had to be able to stay for 60-120s on the rod (cf. *supra*, Animal training).

### 2.3.6 *Weight-bearing distribution*

To assess the changes in hindpaw weight-distribution, an incapacitance tester (Linton instrumentations, UK) was used. The test consisted of placing the rat into a purpose-designed perspex chamber, which allowed the rat to stand up so that the weight distributed on the right and left hindpaws could be measured. Three consecutive readings (each averaging the weight borne on each side over a three-second period) were recorded and later averaged.





Figure 2.5: The Linton incapitance tester instrument.

This apparatus was not available for the first studies. It was thus mainly used in Chapter 7, the AS006 study.

### 2.3.7 *Testing sequence*

**In summary, the behavioural testing sequence was as follows:**

Acetone (1) – VF 1 g – Acetone (2) – VF 6 g – Acetone (3) – VF 8 g – Acetone (4) – Acetone (5) – Rotarod (pain score and latency) – Incapacitance tester (if applicable).

### 2.3.8 *Pharmacological studies*

Various pharmacological agents were tested on this model. The injection protocols will be detailed in the corresponding methods sections.

### 2.3.9 *Data analysis*

Mean values  $\pm$  SEM for each behavioural parameter were determined for each treatment group for each time point. Statistical analyses using the Mann-Whitney U-test with Statview 4.5 software (Abacus Concepts Inc.) were performed to compare responses from control groups with each of the treatment groups for non-parametric data from the mechanical sensitivity, cooling allodynia and rotarod tests. An analysis of variance for repeated measures was applied to the weight-bearing test data (2-way ANOVA, Prism 4, GraphPad Software Inc, California, USA) followed by Bonferroni's post-test comparisons with the vehicle control group as appropriate.

Significance levels were set at  $P \leq 0.05$  (\*),  $P \leq 0.01$  (\*\*) and  $P \leq 0.001$  (\*\*\*).

## 2.4 *In vivo* electrophysiology

### 2.4.1 *Animal preparation*

The *in vivo* electrophysiological recordings were performed as previously described (Svendsen *et al.* 1999a; Suzuki *et al.* 2000; Urch and Dickenson 2003) and as shown on Figure 2.7.A. Animals were anaesthetised with halothane (induction at 3.5%, surgery at 2.0-2.5% and maintenance at 1.0-1.2%), using a 33% oxygen and 66% nitrous oxide mix. After induction of anaesthesia, a tracheotomy was performed, followed by the insertion of a cannula connected to the anaesthesia circuit. The animal was then positioned on a stereotaxic frame. A laminectomy at the L1-L3 vertebral level could then expose the L4-L5 spinal cord segments. The meninges were also removed and the spinal cord fixed by two clamps positioned rostrally and caudally to the site of laminectomy.

### 2.4.2 *Spinal cord recordings*

- 
1. AC recording system with spike discrimination and audio monitoring (NeuroLog system, Digitimer, UK)
  2. Oscilloscope
  3. CED 1401 Interface and Spike 4 software (Cambridge Electronic Design, UK)
  4. Electrodes (Parylene-C insulated tungsten microelectrodes, 125  $\mu\text{m}$  diameter, 2 M $\Omega$ , A-M Systems Inc., Carlsborg, USA)
  5. Three-axis micromanipulator
- 

Table 2.3. Electrophysiological recording equipment required (Adapted from C. E. Urch and A. H. Dickenson, 2003).

Briefly, extracellular recordings of dorsal horn neurones were made using a parylene coated tungsten electrode (A-M systems, Washington). Data was captured and analysed by a CED 1401 interface (Cambridge Electronic Design, Cambridge, UK) coupled to a Pentium computer with Spike 2 software (Figure 2.6).

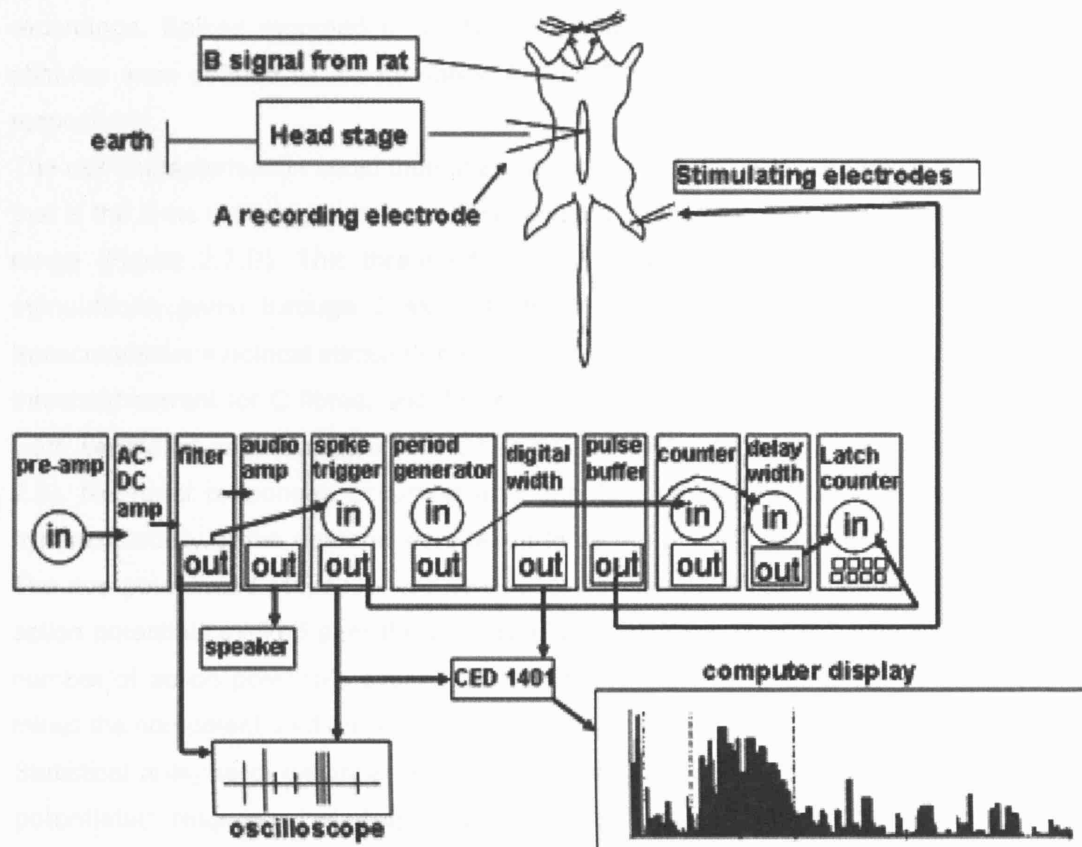


Figure 2.6. Schematic representation of data capture. The recording electrode is inserted into the spinal cord (A), and the animal and stereotaxic frame is grounded (B and earth). The signal is fed into the 'Neurolog', data capture system, where the neuronal signal is amplified, filtered and fed to audio speakers and oscilloscope. The action potentials above set amplitudes are discriminated and fed into the computer system via the CED 1401. Electrical stimuli can be administered via the stimulating electrodes in the neuronal receptive field. The frequency of stimulation, duration, amplitude of the current and number of pulses are set in the period generator, digital width, pulse buffer and counter. The number of action potentials evoked for a set delay width (i.e. adult 90–800 ms), are displayed on the latch counter, and on the computer display (for example as a PSTH, as shown above). Natural stimuli can also be applied with increasing intensity to the receptive field and again the resulting action potentials will be displayed visually on a rate histogram and as numerical action potentials per set time frame (usually 10 s) (C. E. Urch and A. H. Dickenson, 2003).

#### 2.4.2.1 Characterisation of neuronal responses to peripheral electrical stimuli

The electrode was lowered into the spinal cord in order to isolate WDR neurones from deeper laminae (500–900  $\mu\text{m}$ ), with receptive fields (RF) corresponding to the middle and distal parts of the left paw. WDR neurones could be identified by recording a greater response to an increasing tactile force, with a greater response to pinch than to pressure. Checking the amplitude and the shape of the action potentials evoked ensured single cell

recordings. Spikes recorded 0-10, 10-25, 25-300 and 300-800 ms after an electrical stimulus were defined as A-beta fibres, A-delta fibres, C fibres and post-discharge (PD), respectively.

The cell characterisation could then start, by the C-fibre activation threshold measurement that is the 2-ms rectangular pulse current required to elicit 3 action potentials in the C-fibre range (Figure 2.7.B). This threshold was then used for the following electrical skin stimulations given through 2 skin electrodes on the right hindpaw. A train of 16 transcutaneous electrical stimuli (2-ms wide pulses, 0.5 Hz) was applied at three times the threshold current for C fibres, and the responses evoked by A-beta, A-delta and C-fibres were separated and quantified on the basis of their latency following the stimulus (Figure 2.8). Neuronal responses occurring after the C fibre latency band resulting from the hyperexcitability of the neurone were taken to be the post-discharge (PD) of the neurone. The non-potentiated response was calculated as: non-potentiated response = number of action potentials evoked after the first stimulus x 16. Wind-up was calculated as the total number of action potentials evoked at three times the C fibre threshold after 16 stimuli, minus the non-potentiated response.

Statistical analysis of electrical responses (A-beta, A-delta, C fibre, post-discharge, non-potentiated response, wind-up) was conducted using Student's t-test to compare responses from MIA and sham animals.

#### 2.4.2.2 Characterisation of neuronal responses to peripheral natural stimuli

Each test consisted of three 10-second long mechanical and thermal stimuli (Figure 2.7.C): Brush (Waterstones, UK), Von Frey filaments providing forces of 2, 6, 8, 15, 26 and 60 g (Touch-test™ from North Coast Medical Inc., San Jose CA, USA) and constant water jets set at 35, 40, 45 and 48 degrees Celsius (applied with a syringe fitted with a 21 gauge needle).

Statistical analysis comparing the MIA and sham groups was conducted using one-way ANOVA.

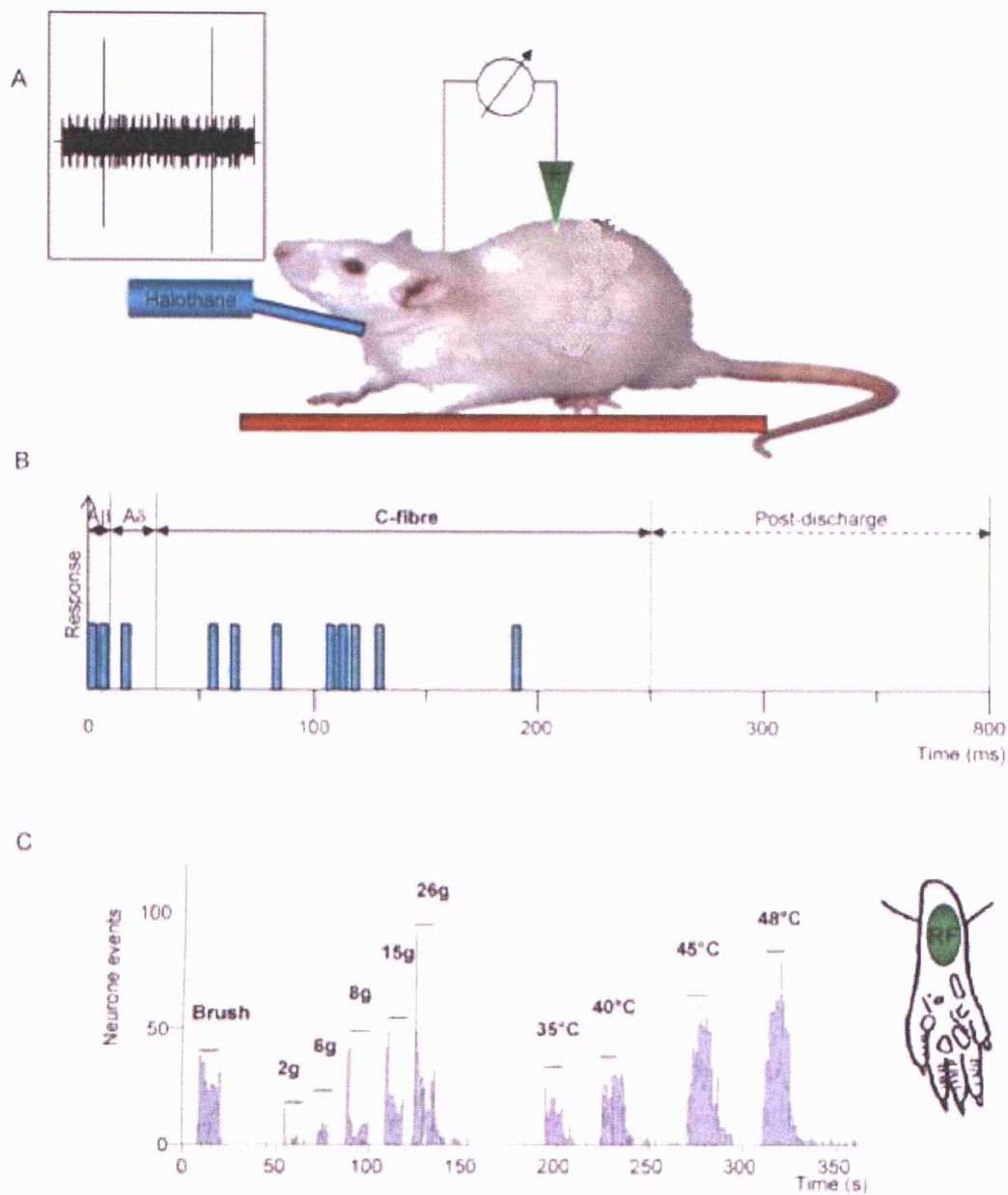


Figure 2.7. Spinal cord lamina V wide dynamic range (WDR) neurones recordings.

(A) Schematic WDR neurone recording made through the electrode E, following a low intensity stimulus applied on the paw. (B) Schematic typical response following an electrical stimulation of the hindpaw. (C) Typical response recorded during the natural stimuli testing: increasing Von Frey filaments force (in grams) and water jets temperature, applied on the receptive field (RF) of the paw.

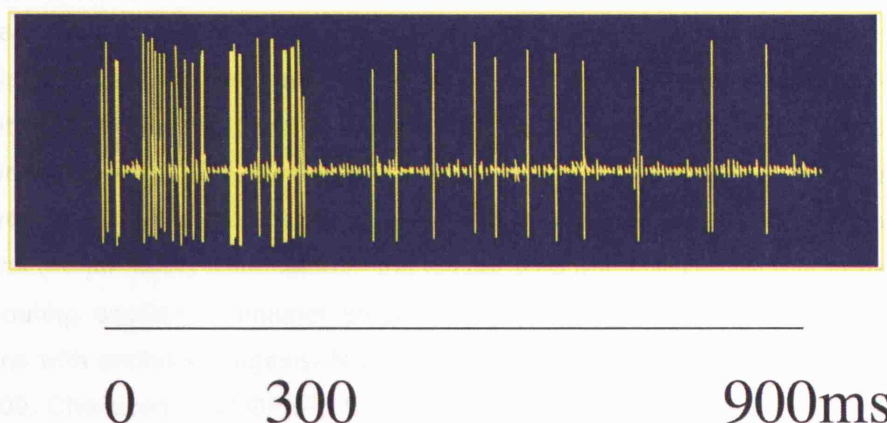


Figure 2.8. Example of raw data recorded for 800 ms following an electrical stimulation in the hindpaw. Each action potential evoked during that period is counted and classified into different types according to its latency following the stimulation: action potentials in the ranges of 0-10, 10-25, 25-300 and 300-800 ms after an electrical stimulus were defined as A-beta fibres, A-delta fibres, C fibres and post-discharge (PD), respectively. The data was then computed into histograms that summed up the responses following the 16 stimulations in order to assess wind-up as shown on Figure 2.6.

## 2.5 Intrathecal drug administration

Intrathecal administration of the substance P-saporin (SP-SAP) toxin was adapted from previously described protocols (P. W. Mantyh et al., 1997; R. Suzuki et al., 2002). Rats (130–150 g) were anaesthetised using 2% isoflurane in oxygen (1 l/min). A small incision was made in the atlanto-occipital membrane and the cannula was inserted into the subarachnoid space, terminating in the L4–5 region. 10  $\mu$ l of SP-SAP ( $10^{-6}$  M, Advanced Targeting Systems, San Diego, California), SAP ( $10^{-6}$  M, Advanced Targeting Systems) or saline were injected using an intrathecal cannula attached to a 25  $\mu$ l Hamilton syringe and flushed with 5  $\mu$ l saline. The cannula was withdrawn, haemostasis confirmed, the muscle layer followed by the skin were sutured using synthetic 3-0 absorbable surgical sutures (Dexon II, Tyco Healthcare Group LP, Norwalk, Connecticut, USA).

These intrathecal injections usually took place 14 days before OA induction. Behaviour was then tested using the previously described techniques. Some electrophysiology was also performed on some animals, as previously described.

Twenty-eight days after the intrathecal injections, two animals belonging to the same batches of used in this experiment were used to verify the actual depletion of SP receptors. This was performed by technicians in the lab. Briefly and as previously described (R. Suzuki et al., 2002), they were transcardially perfused with 4% paraformaldehyde, 0.05% picric acid in PBS (0.15 M, pH 7.4) at 10°C. The spinal cord was removed and cryoprotected overnight in a 30% sucrose solution. Transverse frozen sections (40 µm thick) were cut from the lumbar cord and collected in four serial groups of free-floating sections. Immunohistochemistry reactions were carried out on parallel sections with antibodies against NK1 (1:10,000, Chemicon, Temecula, California), NeuN (1:1,000, Chemicon) and GFAP (1:1,000, Dako, Denmark), using biotinylated secondary antibodies followed by avidin-conjugated fluorescent antibodies, usually Cy3 or FITC. The results were similar to previous studies (R. Suzuki et al., 2002).

## **2.6 Immunohistochemistry**

### **2.6.1 *Retrograde labelling***

In order to label in the DRGs the primary afferents coming from the knee, I used the fluorescent neuronal marker Fast Blue (FB) that would be taken up at the nerve terminals in the knee and transported up to the cell bodies.

FB was administered intraarticularly as for the previously described MIA injection, either 6 days before (10 µl of 1% FB) or at the same time as the MIA injection (10 µl of 1% FB were mixed with 2 mg of MIA and 15 µl of saline, reaching a final injection volume of 25 µl).

In order to limit the number of animals, contralateral knee joints were used as controls and were injected on the same day with the same volume of saline only (either 10 µl or 25 µl depending on the experiment).

### 2.6.2 *Perfusion and tissue preparation*

14 days post-intraarticular injection, following the last behavioural time point, animals were terminally anaesthetised with pentobarbital sodium (200 mg in 1 ml, i.p.). The thoracic cage was then opened, the heart exposed and freed from the pericardium. Animals were transcardially perfused with 200 ml heparinised saline (5000 IU of heparin sodium in 1 litre of 0.9% w/v NaCl) followed by 400 ml of 4% w/v paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The lumbar DRGs L3, L4 and L5, right (IL) and left (CL), were dissected out and post-fixed in the same solution for another 2 h. They were then cryoprotected in 30% w/v sucrose in 0.1 M PB for a minimum of 24 h at 4°C. Tissues were embedded in OCT embedding compound (BDH). Transverse sections were cut serially (12 µm thickness) on a cryostat and mounted onto Superfrost slides (BDH). Every section of the DRGs was collected on a series of 6-8 slides: each section was collected on a different slide (between 6 and 8 slides for smaller and larger DRGs respectively) in order to have an overall representation of the DRG neuronal population on each slide and to eventually allow various markers to be tested.

Sections were processed for immunohistochemistry using triple labelling of Fast Blue, ATF-3, and beta-III tubulin, following previously described protocols (S. Pezet et al., 2006; P. J. Shortland et al., 2006). Briefly, after several washes in phosphate-buffered saline (PBS; 0.01 M PB, 0.9% NaCl, pH 7.4), sections were incubated overnight with primary antibodies. After three washes in PBS, they were incubated 2 h with secondary antibodies and then covered with Vectashield medium (Vector Laboratories). Details about the antibodies used are given in the table below.

Type of labelling	Primary antibodies	Secondary antibodies
<b>ATF3</b>	Rabbit anti-ATF-3 (1:500; Santa Cruz)	Goat anti-rabbit Alexa Fluor 488 (1:1000; Molecular Probes)
<b>Beta-III tubulin</b>	Mouse anti-beta-III tubulin (1:2000; Promega)	Goat anti-mouse Alexa Fluor 546 (1:1000; Molecular Probes)

Table 2.4. Concentrations and details of antibodies used.

To maintain consistency, each type of immunostaining was done simultaneously for the animals we wanted to compare. Omission of the primary antibody or omission of any stage in the protocol did not result in labelling. All antibodies and sera were diluted in 0.01 M



PBS, 0.1% w/v sodium azide (only for primary antibodies, overnight incubation), and 0.3% v/v Triton X-100. Incubation of slides was done at room temperature.

Sections were viewed under an Axioplan 2 Imaging microscope (Imaging Associates) fitted with 10-time, 20-time, and 40-time Plan-Neofluoro objectives (Zeiss, Germany) and images were taken using an AxioCam Hrm digital camera (Zeiss, Germany) and AxioVision software (Imaging Associates).

### **2.6.3 Quantification of ATF-3-positive neurones in the DRG**

Quantification of ATF-3-positive neurones was done by counting the number of positive and negative cells with visible nuclei on at least one slide displaying a representative image of the neuronal population (through a series of DRG slices corresponding to every 6 or 8 sections depending on the DRG) in a given DRG for a given animal. The number of cells positive for ATF-3, FB and beta-III tubulin was counted in images acquired using a 40-time objective. The total number of cells was manually counted using the beta-III tubulin staining as a neuronal marker, counting only the cells with visible nuclei. Results are expressed as the percentage of ATF-3-positive cells in all cells of the DRG. Variations between groups were statistically analysed using one-way ANOVA on ranks, followed by Tukey post-hoc test.

**Chapter 3. Effects of morphine and gabapentin following the validation  
of the MIA-induced model of osteoarthritis pain.**

### 3.1 Introduction

One in five adults is suffering from chronic pain and among those, arthritis was the primary cause of pain for more than 30% of respondents (H. Breivik et al., 2006). Osteoarthritis (OA), the most common form of arthritis (WHO, 2003; D. T. Felson, 2004), is a degenerative joint disease associated with chronic debilitating joint pain.

Whether it affects the hands or any weight-bearing joint such as the knee, the hip, the back or the feet, the current pharmacological treatments are only symptomatic - aiming at pain relief using analgesics or anti-inflammatory drugs - as no disease modifying osteoarthritis drugs (DMOADs) are available.

Preclinical models of OA could be very useful in order to better understand the pathophysiology of the disease and to test for new therapeutic options. Following a single intra-articular MIA injection, progressive cartilage degeneration caused by the inhibition of chondrocytes' metabolism was first shown in hens (D. A. Kalbhen and U. Blum, 1977) then studied in rats (D. A. Kalbhen, 1987).

Histopathological studies showed changes similar to the human disease (C. Guingamp et al., 1997; M. J. Janusz et al., 2001). Inflammation was found to be resolved after day 7 within the synovium and surrounding tissues (S. E. Bove et al., 2003). The model was then qualified as a "model of chronic nociceptive pain in rats" (R. Combe et al., 2004) with an increased robustness in comparison to the partial medial meniscectomy surgical model of OA (J. Fernihough et al., 2004) or to the chemical papain model of OA (J. D. Pomonis et al., 2005).

Some pharmacological studies have been performed at various time points using different NSAIDs and analgesics such as morphine and gabapentin (R. Combe et al., 2004; J. Fernihough et al., 2004; J. D. Pomonis et al., 2005; S. P. Ivanavicius et al., 2007) and showed various degrees of efficacy. However, these were only given single doses but not repeated ones, as it would be the case for human patients.

In this study, I first wanted to validate the MIA-induced model of OA pain with new behavioural tests. I then performed a pharmacological modulation of the model using both acute and chronic administrations of morphine and gabapentin.

## **3.2        *Methods***

These have been described extensively in Chapter 2 (Methods) and some details are given here.

### **3.2.1   *Animals***

Male Sprague-Dawley rats (Central Biological Services, University College London) weighing 120-140 g at the time of surgery for the induction of the model (day 0) were used. They were randomly housed at a maximum of five per cage on a 12h day-12h night cycle with food and water available ad libitum. All experimental procedures were approved by the UK Home Office and follow the guidelines of the International Association for the Study of Pain (M. Zimmermann, 1983).

### **3.2.2   *Animal training***

4 days before the surgery (referred as day -4), animals were selected on both weight and rotarod aptitude criteria. The rotarod tests will be detailed in the behaviour section. Briefly, only rats presenting no aversion for the device, i.e. that could stay on the rotating rod for at least 60 seconds before falling down, and showing normal locomotive function were used.

### **3.2.3   *Induction of the MIA model of OA***

On day 0, after clipping the hairs covering the patellar area, animals were anaesthetised using halothane (4% for induction, 2% for maintenance) in oxygen (1 litre/min) and placed in the supine position. After disinfecting the skin with a povidone-iodine solution (Betadine<sup>TM</sup>), a single injection of 25  $\mu$ l sterile 0.9% sodium chloride (NaCl, saline) containing 2 mg of monosodium iodoacetate (MIA, Sigma, UK) was administered through the patellar tendon (also known as the infra-patellar ligament) of the right (ipsilateral, IL) knee using a 27G needle. Sham animals received sterile 0.9% NaCl only. Contralateral (CL) knees did not receive any treatment.

For the validation study, MIA and saline injections were blinded and randomised.

#### 3.2.4 *Behavioural studies*

Behaviour was assessed at different time points following OA induction in a blinded and randomised manner, for up to 18 days.

Following a 30-minute acclimatisation period, the behavioural tests started, investigating cooling and mechanical hypersensitivity of the hindpaws, as well as ambulatory-evoked pain.

Cooling sensitivity was assessed using the acetone drop test: a drop was applied to the plantar surface of the hindpaws, both ipsilateral and contralateral to the injected knee. A marked, delayed, flinching, shaking or toe-biting behaviour was scored positive. The test was repeated a total of 5 times on each side with a minimum of 2 min. between each application.

Mechanical hypersensitivity was assessed using von Frey filaments (Touch-test™, North Coast Medical Inc., San Jose CA, USA) applied on the plantar surface and the toes of the hindpaws, with forces of 1, 6 and 8 g, respectively 9.8, 58.9 and 78.5 mN. Each von Frey filament was applied 10 times over the plantar surface and the toes on the IL and CL paws; they were tested between the acetone tests. Withdrawal responses and whole paw lifts elicited by the von Frey hairs were scored as positive.

An accelerating Rotarod device (Rotarod 7750, Ugo Basile, Italy) was used to assess ambulatory-evoked pain. The apparatus was set to accelerate from 0 to 20 revolutions per minute (rpm) over 60 sec and the latency to fall was monitored with a maximum cut-off of 180 sec. The limp was also scored from 0 to 3 as follows: 0: no limp, 1: slight limp but no decreased usage of the IL limb, 2: limp with decreased usage of the IL limb, 3: avoidance of use of the IL limb.

#### 3.2.5 *Pharmacological studies*

All the animals used had OA induced with MIA 14 days, and the development of a chronic nociceptive state was checked before starting the pharmacological study on day 14, once the baseline responses were recorded.

From day 14 to day 17, animals then received chronic bi-daily subcutaneous (SC) injections (at 8 am and 6 pm) of either 3 mg/kg morphine (morphine sulphate, Thornton and Ross) or gabapentin 30 mg/kg (a gift from Pfizer, Sandwich, UK) in 0.9% NaCl, with a final injection on the morning of day 18. Control animals received NaCl alone, with

injection volumes equivalent to 1 ml/kg. During that period, behavioural tests started 30 min after the morning (8 am) injection.

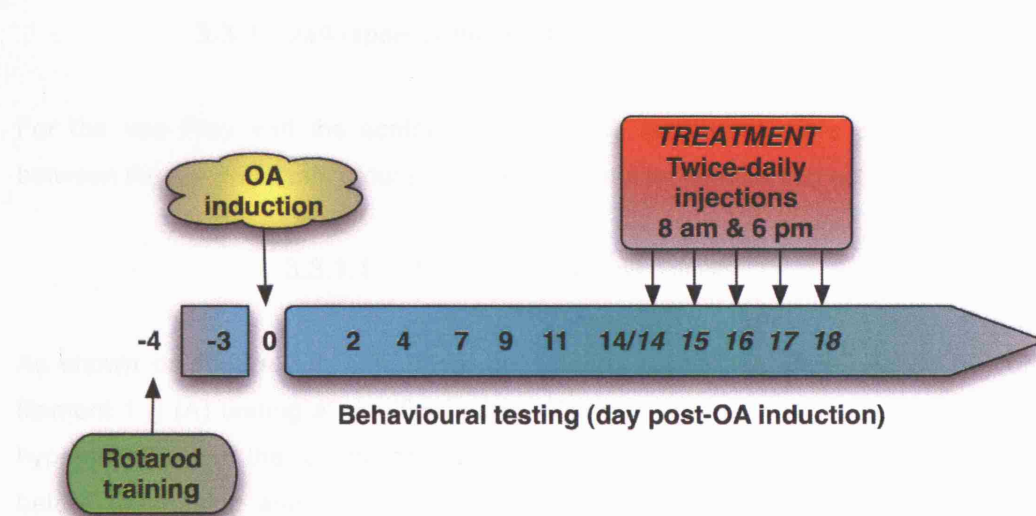


Figure 3.1. Diagram of the experimental time-course. On day 14, baseline responses were first recorded, then the treatment was administered and the responses recorded again 30 min post-injection. On the other days of the treatment period, testing started 30 minutes after the 8 am injection.

### 3.2.6 Data analysis

Data are presented as mean  $\pm$  SEM. Statistical analyses were performed using the Mann-Whitney U-test (comparisons between conditions: MIA or sham, and between treatments: drug or control groups) and Wilcoxon matched pairs test (acute effect of drug on day 14) using GraphPad Prism (version 4.0c for Mac, GraphPad Software, San Diego California USA) and significance levels set at  $P \leq 0.05$  (\*),  $P \leq 0.01$  (\*\*) and  $P \leq 0.001$  (\*\*\*).

### 3.3 Results

#### 3.3.1 *Validation of the model*

For the von Frey and the acetone tests, no CL responses were statistically different between the OA and sham groups. They will thus not be commented upon.

##### 3.3.1.1 Mechanical hypersensitivity

As shown on Figure 3.2, only minor behavioural responses were evoked by von Frey filament 1 g (A) testing at any time point. With von Frey 8 g (C), a biphasic mechanical hypersensitivity of the IL limb appeared first on day 2 following the injection ( $p=0.0002$ ), before decreasing and reaching a stable plateau on days 11 and 14 ( $p=0.0168$  and  $p=0.0123$  respectively), whereas with von Frey 6 g (B), the two phases were less pronounced: the first response peak was delayed until day 4 ( $p=0.0253$ ) and the late development of the mechanical hypersensitivity only clearly occurred from day 14 ( $p=0.0087$ ).

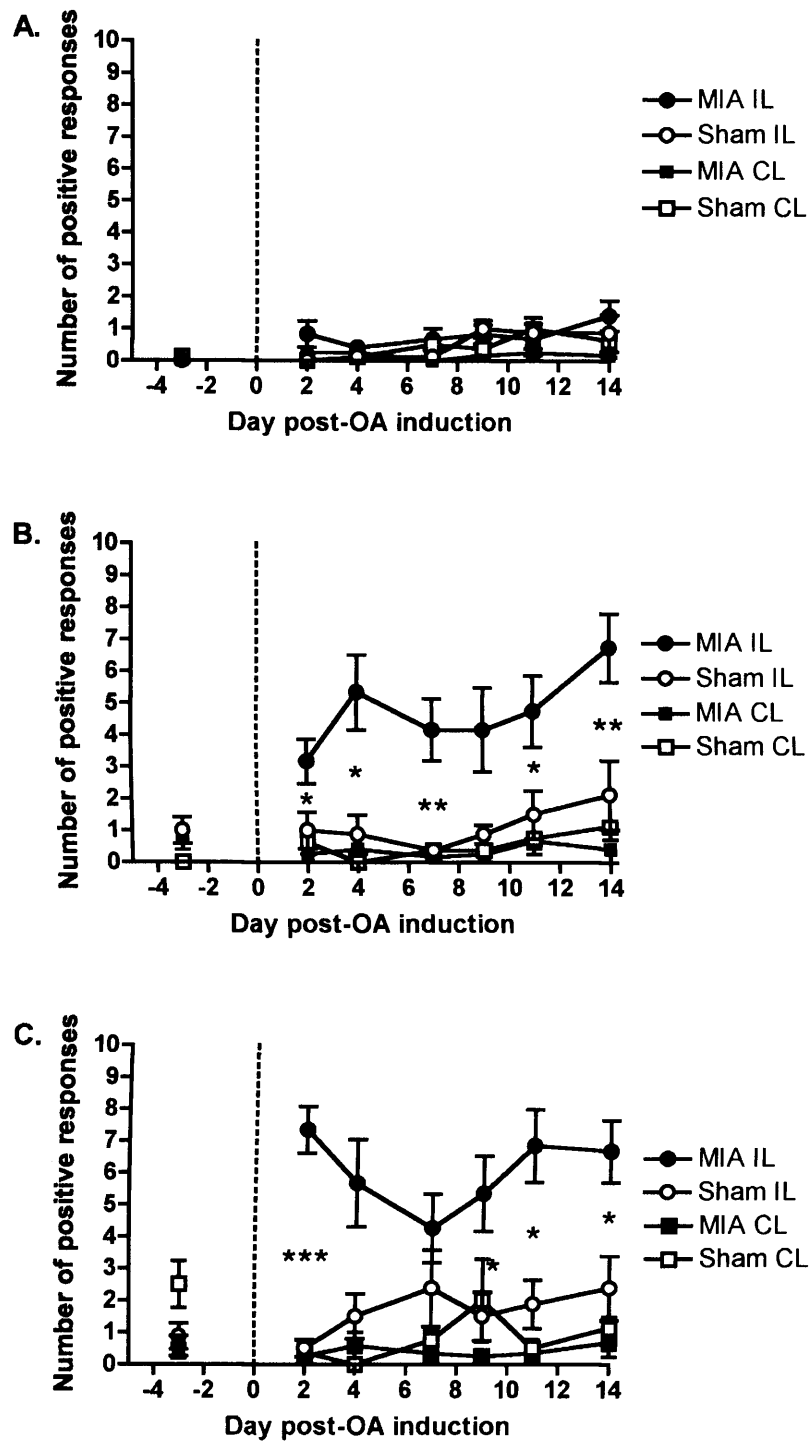


Figure 3.2. Development of mechanical hypersensitivity following OA induction. Von Frey filaments 1, 6 and 8 g (A, B and C respectively) were applied 10 times over the plantar surface of each hindpaw – ipsilateral (IL) and contralateral (CL) to the MIA injected knee. Data presented as mean  $\pm$  SEM, MIA group  $n=12$ , Sham group  $n=8$ , comparisons using Mann-Whitney U-test. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ .



### 3.3.1.2 Cooling hypersensitivity

When using the acetone drop test, a cooling hypersensitivity gradually developed after the MIA injection, with a peak difference on day 7 ( $p=0.0186$ ), with close to significantly different levels reached on days 9 and 11 ( $p=0.0538$  and  $p=0.0641$  respectively) followed by a clear trend towards a long-lasting increased hypersensitivity.

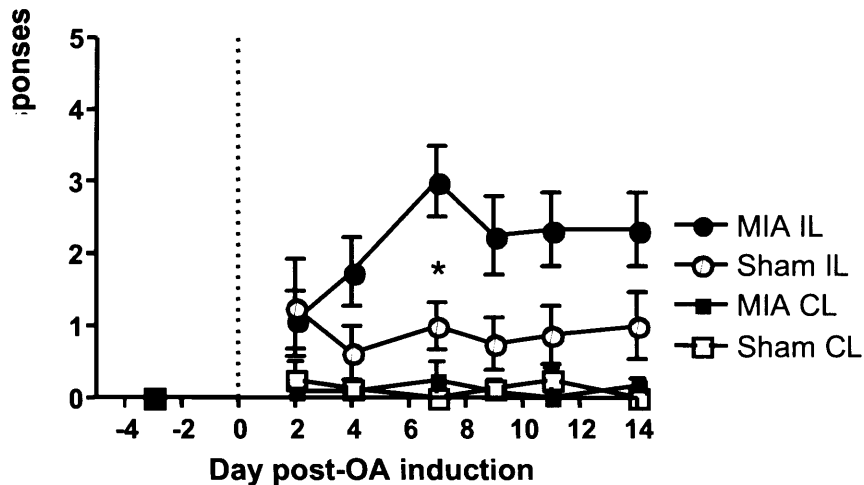


Figure 3.3. Development of cooling hypersensitivity following OA induction. A drop of acetone was applied a total of 5 times over the plantar surface of each hindpaw -ipsilateral (IL) and contralateral (CL) to the MIA injected knee. Data presented as mean  $\pm$  SEM, MIA group  $n=12$ , Sham group  $n=8$ , comparisons using Mann-Whitney U-test.  $*P\leq 0.05$ .

### 3.3.1.3 Ambulatory-evoked pain score

As expected, the ambulatory-evoked pain score was null in both MIA and sham groups before the induction of the model. Once the model was induced, a statistically significant score difference of about one unit was consistently maintained throughout the first 9 days of the model characterisation, which increased to a 2-unit difference on day 14 ( $p=0.001$ ).

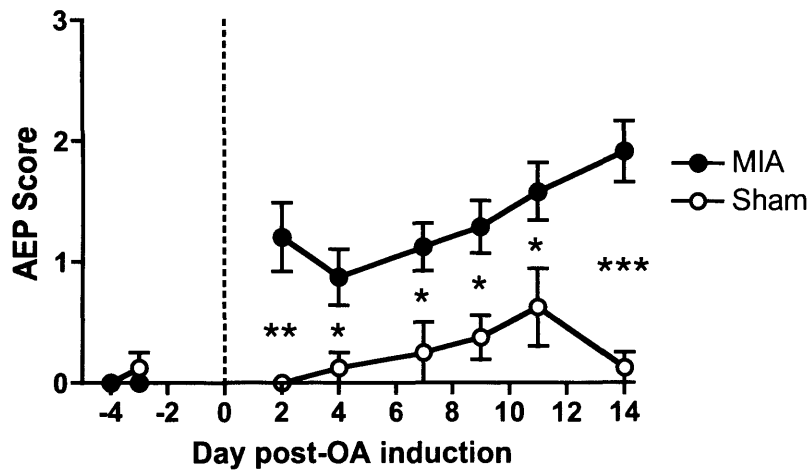


Figure 3.4. Evolution of ambulation evoked pain (AEP) score after OA induction. Using an accelerating rotarod, the AEP was scored on a scale from 0 (normal) to 3 (avoidance of use of limb). Data presented as mean  $\pm$  SEM, MIA group  $n=12$ , Sham group  $n=8$ , comparisons using Mann-Whitney U-test. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ .

#### 3.3.1.4 Latency to fall

Before the induction of the model on days  $-4$  and  $-3$ , as expected, there was no difference between the MIA and the sham groups. After the surgery on day 0, the MIA-treated group managed to stay on the rotarod for a shorter period of time than the control group only on days 2 and 4 ( $p=0.0026$  on both days). From day 7 onwards, there was no significant difference between the two groups.

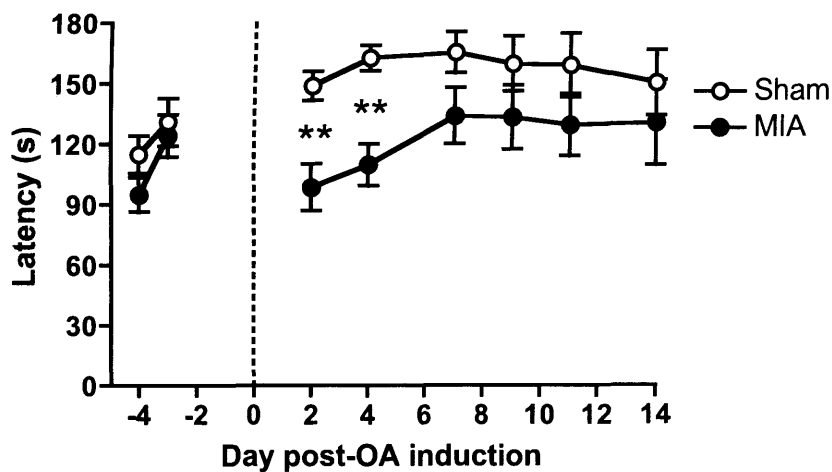


Figure 3.5. Changes in Latency to fall following OA induction. Data presented as mean  $\pm$  SEM, MIA group  $n=12$ , Sham group  $n=8$ , comparisons using Mann-Whitney U-test. \*\* $P \leq 0.01$ .

### 3.3.1.5 Weight gain

During these experiments, the animals' weight curves did not differ, suggesting that the condition induced did not have any major influence on their general health.

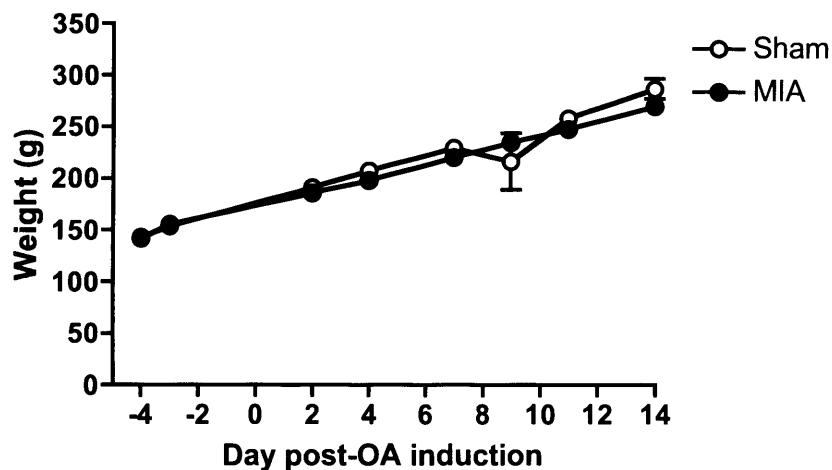


Figure 3.6. Weight gain during the development of the model. Data presented as mean  $\pm$  SEM, MIA group  $n=12$ , Sham group  $n=8$ , comparisons using Mann-Whitney U-test. \* $P \leq 0.05$ .

The small decrease in the sham group on day 9 was caused due to an animal presenting an ingrown tooth, preventing it from feeding properly, which was rapidly fixed. Monitoring the animals' weight on each experimental day is thus confirmed as a simple way to verify their general well-being.

### 3.3.2 *Acute effects of morphine and gabapentin*

Behavioural assessment was performed at various time-points on MIA-injected rats to check the expression of persistent pain behaviour on day 14, in agreement with the previous results. As this was the case, the behavioural data up to day 14, before the administration of either morphine or gabapentin, will not be commented upon.

Comparisons were made both between baseline and post-treatment responses and between vehicle- and morphine- or gabapentin-treated animals.

#### 3.3.2.1 Mechanical hypersensitivity

As shown on Figure 3.7, after the first morphine injection on day 14 the mechanical hypersensitivity was greatly reduced, in a statistically significant manner, when compared to both baseline responses and to vehicle-treated animals with von Frey hairs 6 and 8 g.

The first acute dose (30 mg/kg) of gabapentin did not have any effect on mechanical hypersensitivity (Figure 3.8).

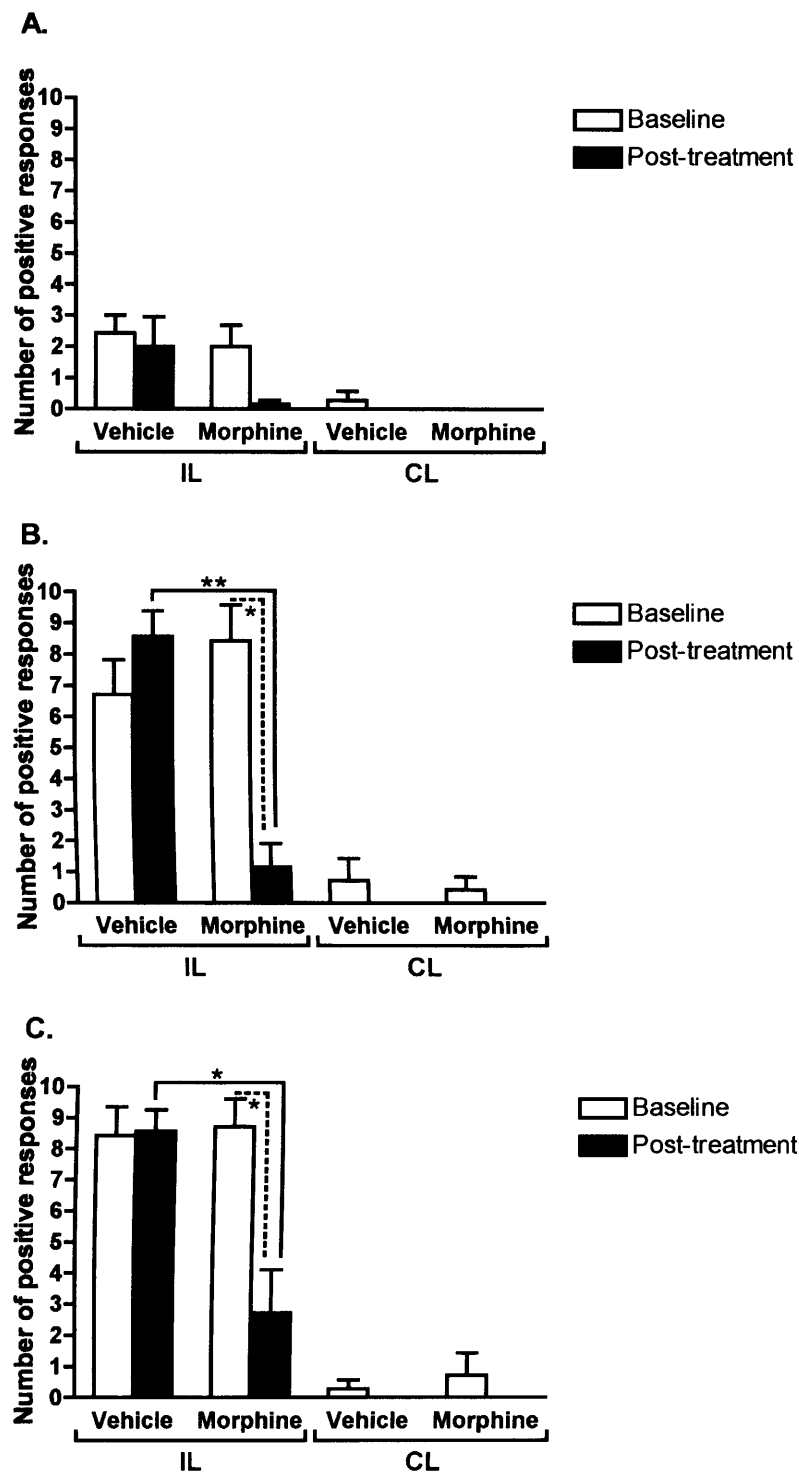


Figure 3.7. Effects of acute morphine 3 mg/kg on mechanical hypersensitivity on day 14, 30 min post-treatment. Von Frey filaments 1, 6 and 8 g (A, B and C respectively) were applied 10 times over the plantar surface of each hindpaw, ipsilateral (IL) and contralateral (CL) to the MIA injected knee. Data presented as mean  $\pm$  SEM,  $n=7$  in each group, comparisons using Wilcoxon matched pairs test for baseline-post treatment comparisons and Mann-Whitney U-test for morphine-vehicle comparisons. \* $P\leq 0.05$ , \*\* $P\leq 0.01$ .

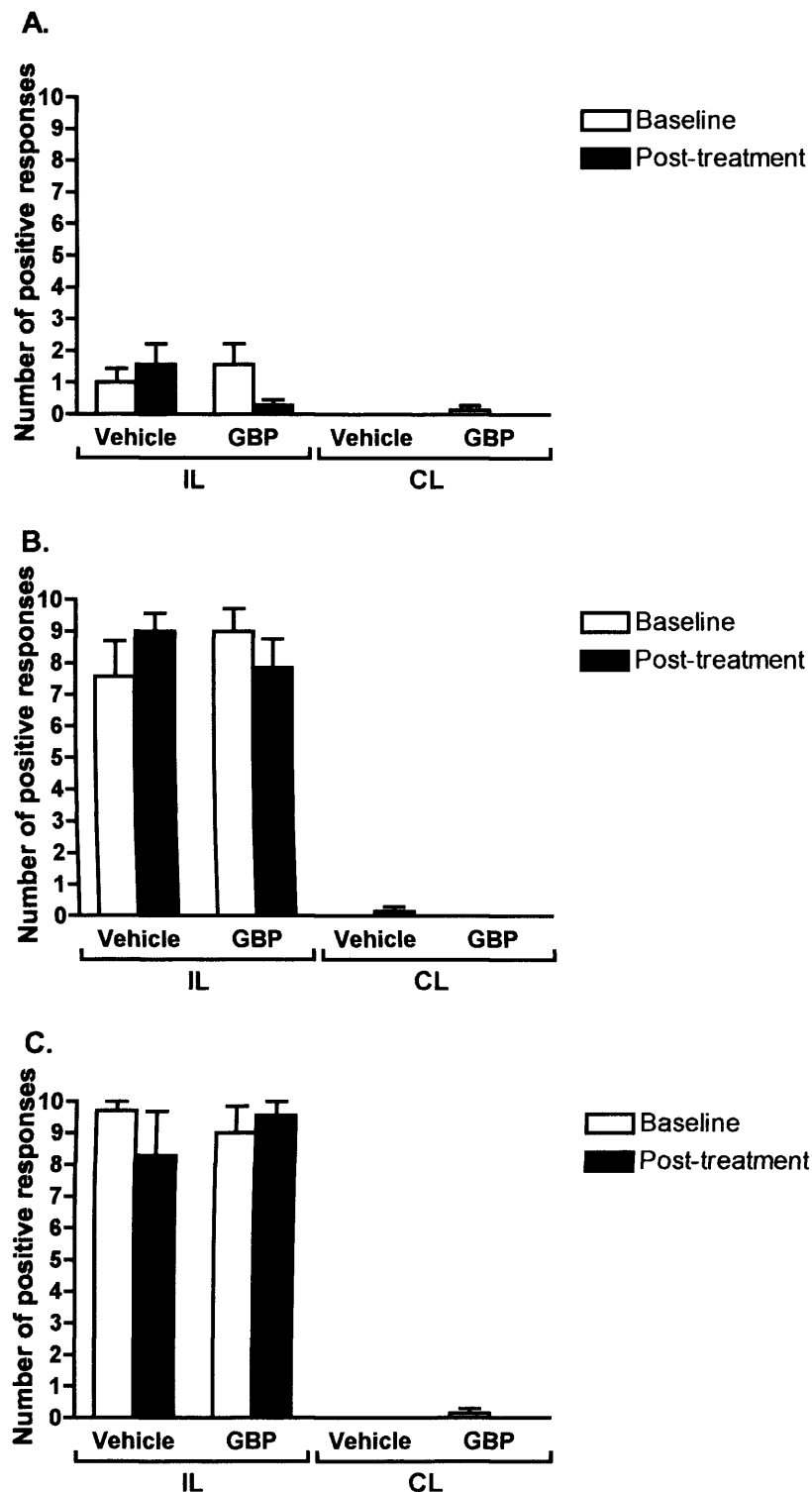


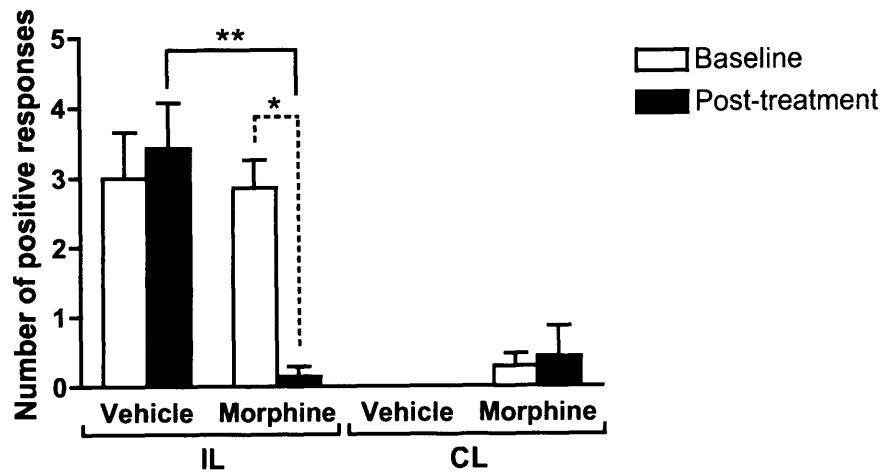
Figure 3.8. Effects of acute gabapentin 30 mg/kg on mechanical hypersensitivity on day 14, 30' post-treatment. Effects of acute morphine on mechanical hypersensitivity on day 14, 30 min post-treatment. Von Frey filaments 1, 6 and 8 g (A, B and C respectively) were applied 10 times over the plantar surface of each hindpaw, ipsilateral (IL) and contralateral (CL) to the MIA injected knee. Data presented as mean  $\pm$  SEM,  $n=7$  in each group.

#### 3.3.2.2 Cooling hypersensitivity

An acute dose of morphine (Figure 3.9.A) induced a very marked statistically significant decrease in the response frequency to the acetone drop test, from a baseline value around 3 out of 5 to 0.5 post-treatment. This difference was also present in comparison to the vehicle-treated group at the same time-point.

Acute gabapentin (Figure 3.9.B) on the other hand, did not induce any significant changes.

A.



B.

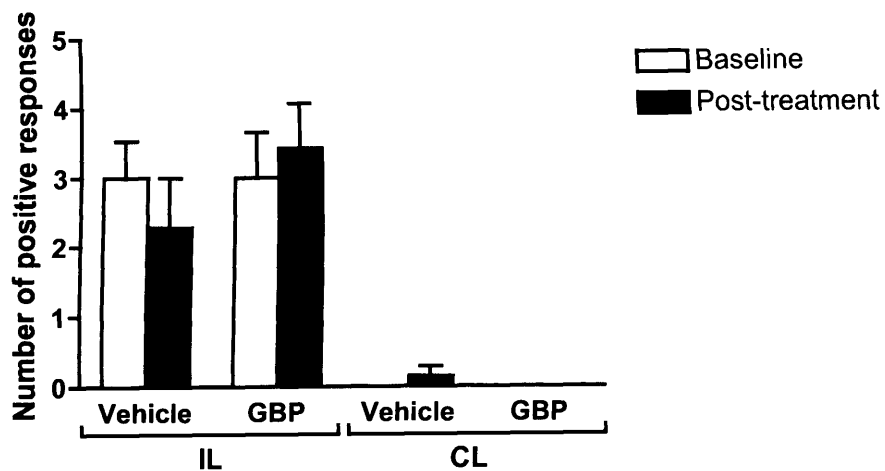


Figure 3.9. Effects of acute (A) morphine 3 mg/kg or (B) gabapentin 30 mg/kg on cooling hypersensitivity on day 14, 30' post-treatment. A drop of acetone was applied a total of 5 times over the plantar surface of each hindpaw -ipsilateral (IL) and contralateral (CL) to the MIA injected knee. Data presented as mean  $\pm$  SEM,  $n=7$  in each group, comparisons using Mann-Whitney U-test. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ .



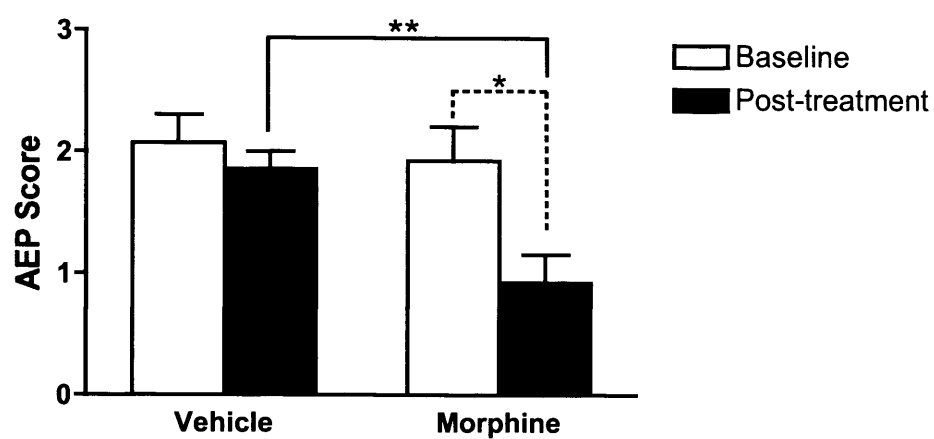
### 3.3.2.3 Ambulation evoked pain score

While the vehicle treatment did not have any effect on the ambulation evoked pain score, morphine did produce a statistically significant 1-unit decrease ( $P<0.05$ ) 30 minutes post-administration (Figure 3.10.A).

As expected, this difference was also statistically significant when compared to the vehicle-treated group at the same time-point ( $P<0.01$ ).

An acute dose of gabapentin (Figure 3.10.B) did not produce any change in the pain score.

A.



B.

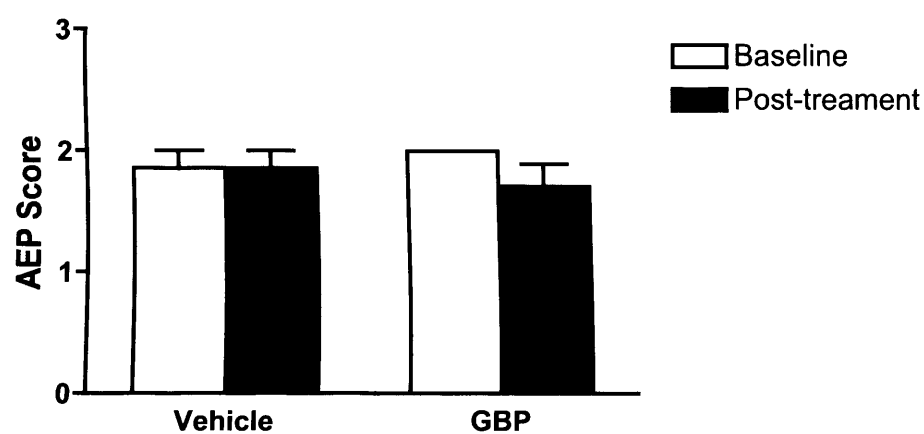


Figure 3.10. Effects of acute (A) morphine 3 mg/kg or (B) gabapentin 30 mg/kg on the ambulatory evoked pain score on day 14, 30' post-treatment, using a rotarod. Data presented as mean  $\pm$  SEM,  $n=7$  in each group, comparisons using Wilcoxon matched pairs test for baseline-post treatment comparisons and Mann-Whitney U-test for morphine-vehicle comparisons. \* $P\leq 0.05$ , \*\* $P\leq 0.01$ .

#### 3.3.2.4 Latency to fall

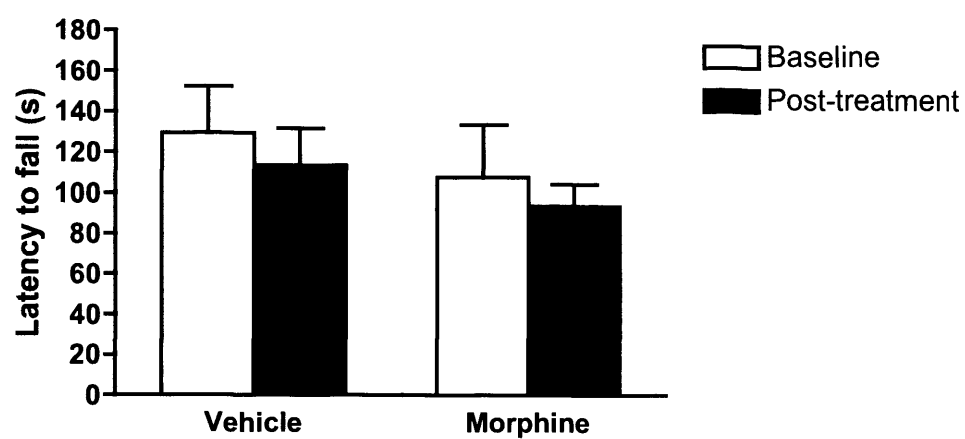
As expected, the vehicle treatment did not have any influence on the latency to fall (Figure 3.11).

Acute morphine 3 mg/kg (Figure 3.11.A) also had no effect.

However gabapentin 30 mg/kg (Figure 3.11.B) provoked a statistically significant decrease (in the range of 30 seconds on average,  $P < 0.05$ ) in the rats' performance of the rotarod.

This effect could suggest that the effects of gabapentin may influence the animals' latency to fall, which will be studied in more detail in the chronic treatment section.

A.



B.

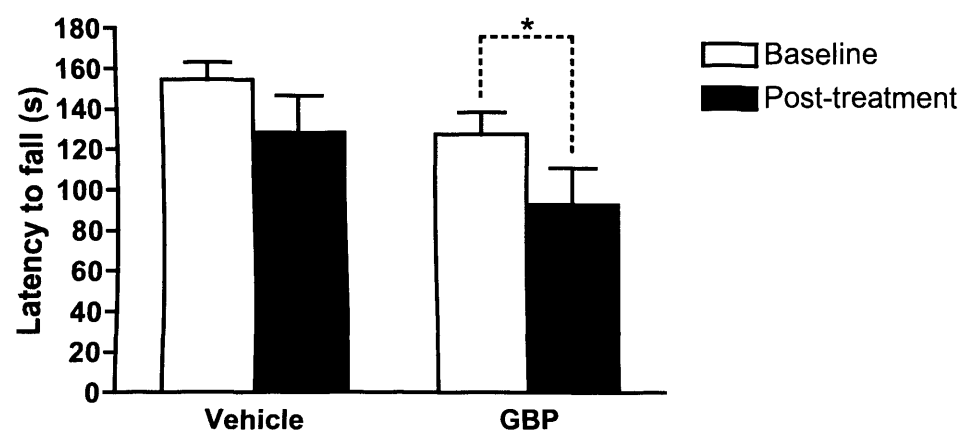


Figure 3.11. Effects of acute (A) morphine 3 mg/kg or (B) gabapentin 30 mg/kg on the latency to fall on day 14, 30' post-treatment, using a rotarod. Data presented as mean  $\pm$  SEM,  $n=7$  in each group, comparisons using Wilcoxon matched pairs test for baseline-post treatment comparisons and Mann-Whitney U-test for morphine-vehicle comparisons. \* $P \leq 0.05$ .

### 3.3.3 *Chronic effects of morphine and gabapentin*

#### 3.3.3.1 Mechanical hypersensitivity

The previous results showed that the injection of morphine had an clear acute effect in reducing the response frequency to von Frey filaments 6 and 8 g. Whereas this mechanical hypersensitivity reduction was maintained over the 5 days of the chronic treatment period, the effects of morphine seemed to gradually diminish with time, ending with no statistically significant difference on day 18 for von Frey 8 g (Figure 3.12.C).

With gabapentin (Figure 3.13), the time-course was different: whereas there was no acute effect on day 14, the von Frey testing revealed a tendency for a decrease in the number of withdrawal responses, with statistically different responses only towards the end of the treatment. Indeed, von Frey hair 6 g (Figure 3.13.B) showed a positive effect on day 18 whilst von Frey hair 8 g (Figure 3.13.C) only had a statistically significant effect on day 17, although day 18 was close to statistical significance.

It was thus interesting to note these opposite effects of morphine and gabapentin, which generally speaking tended to decrease and increase with time, respectively. This time-course difference was very obvious and was in agreement with the drugs' pharmacological properties.

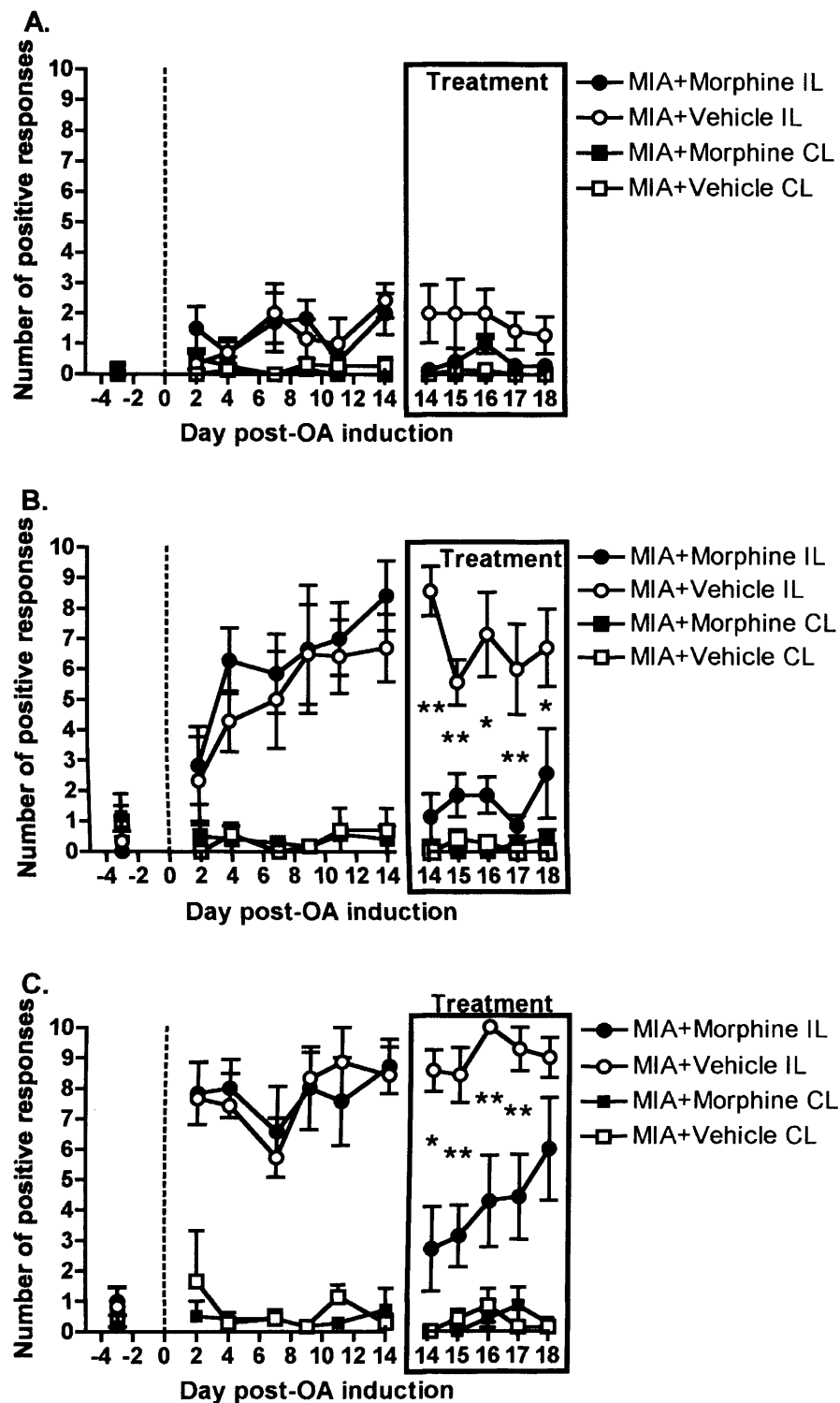


Figure 3.12. Effects of chronic morphine (3 mg/kg twice daily) on mechanical hypersensitivity, 30' post-treatment. Von Frey filaments 1, 6 and 8 g (A, B and C respectively) were applied 10 times over the plantar surface of each hindpaw -ipsilateral (IL) and contralateral (CL) to the MIA injected knee. Data presented as mean  $\pm$  SEM,  $n=7$  in each group, comparisons using Mann-Whitney U-test. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ .

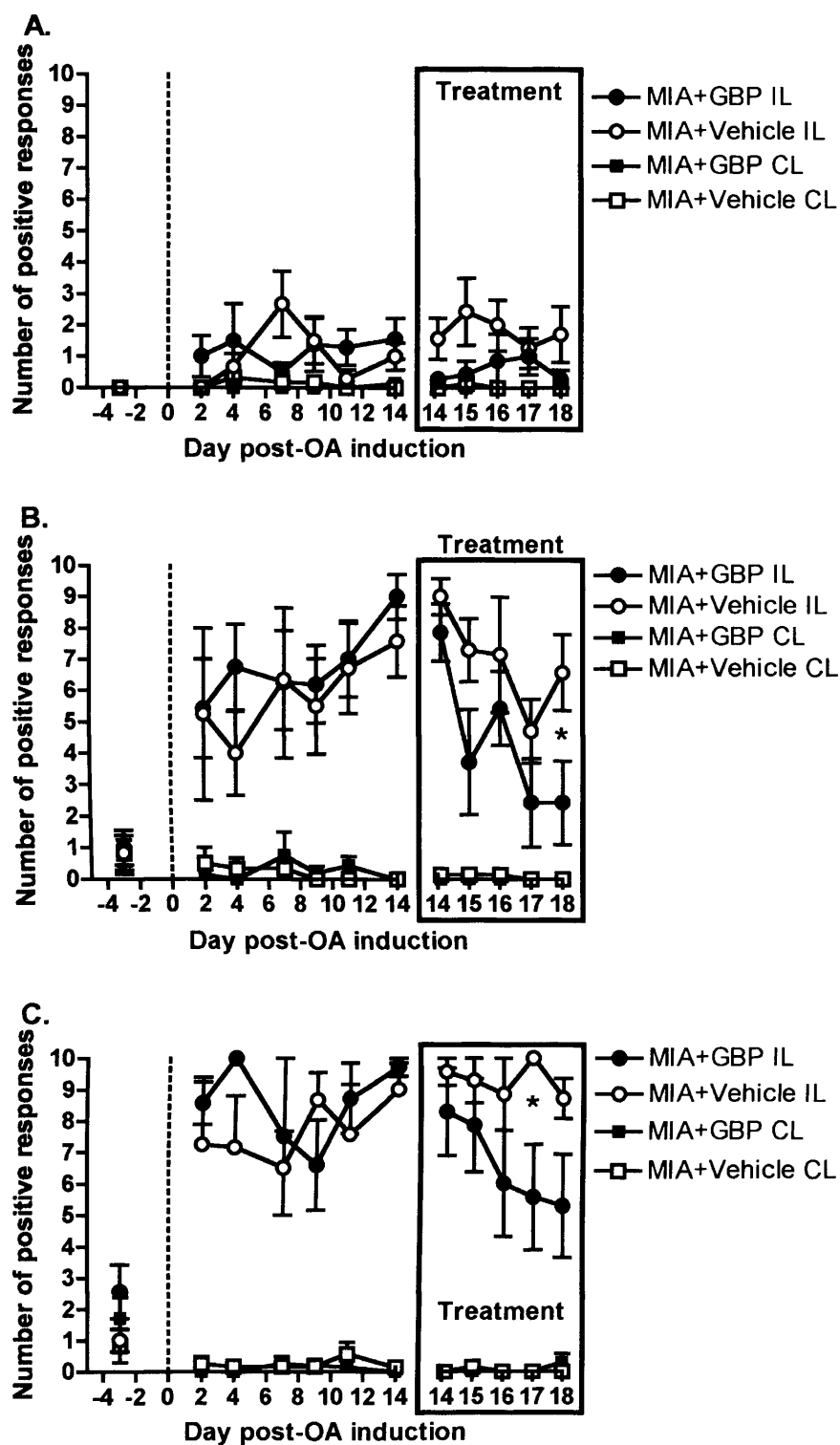


Figure 3.13. Effects of chronic gabapentin (30 mg/kg twice daily) on mechanical hypersensitivity, 30' post-treatment. Von Frey filaments 1, 6 and 8 g (A, B and C respectively) were applied 10 times over the plantar surface of each hindpaw -ipsilateral (IL) and contralateral (CL) to the MIA injected knee. Data presented as mean  $\pm$  SEM,  $n=7$  in each group, comparisons using Mann-Whitney U-test. \* $P \leq 0.05$ .

### 3.3.3.2 Cooling hypersensitivity

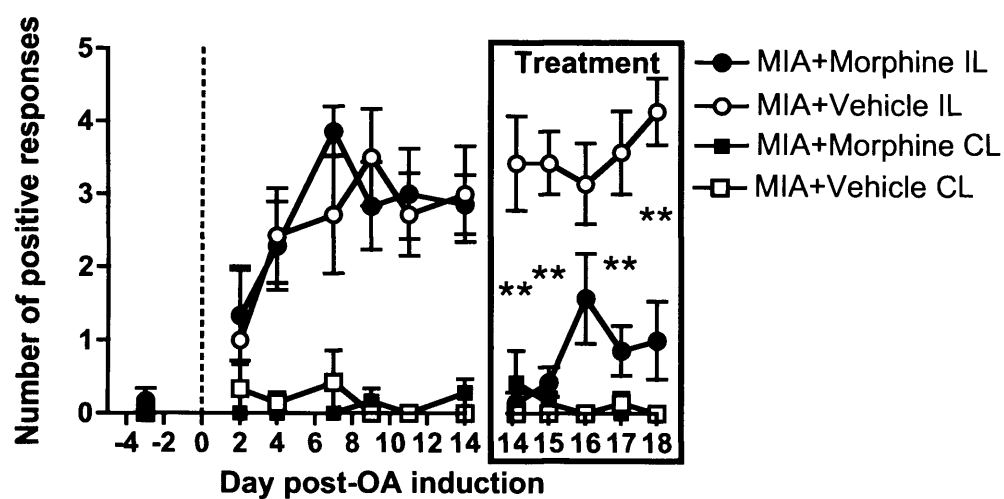
Chronic morphine administration markedly reduced this cooling hypersensitivity with statistical significance reached throughout the 5-day chronic administration, except on day 16 (Figure 3.14.A).

Chronic gabapentin treatment revealed similar patterns but statistical significance was only reached on days 15 and 17 (Figure 3.14.B). There was no difference on day 16, and on day 18, there was a tendency for a difference between the gabapentin-treated and the control groups but it was not statistically significant.

With the acetone drop test, it appeared that morphine might have a stronger effect than gabapentin on cooling hypersensitivity. The reason for the lack of difference between the two groups on day 16 is unclear (although those experiments were made in various blinded and randomised batches) but could be due to a change in cooling sensitivity mechanisms at that time.



A.



B.

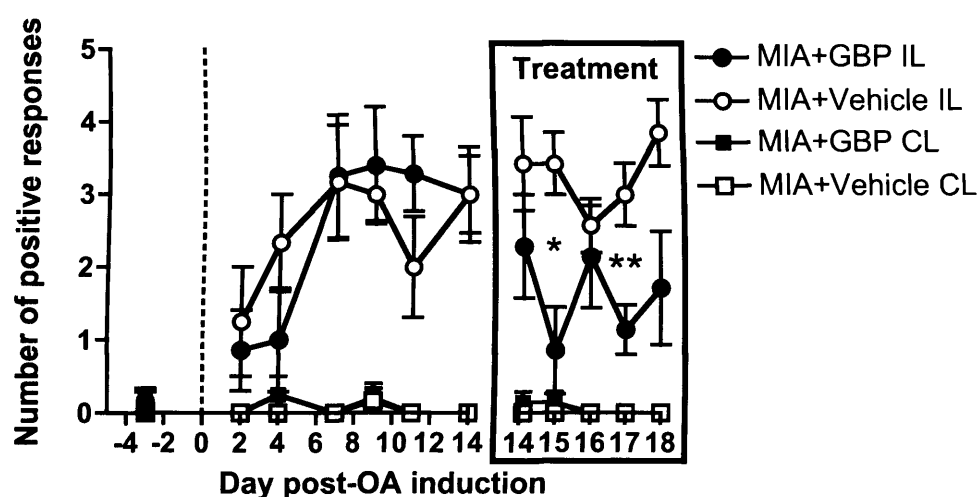


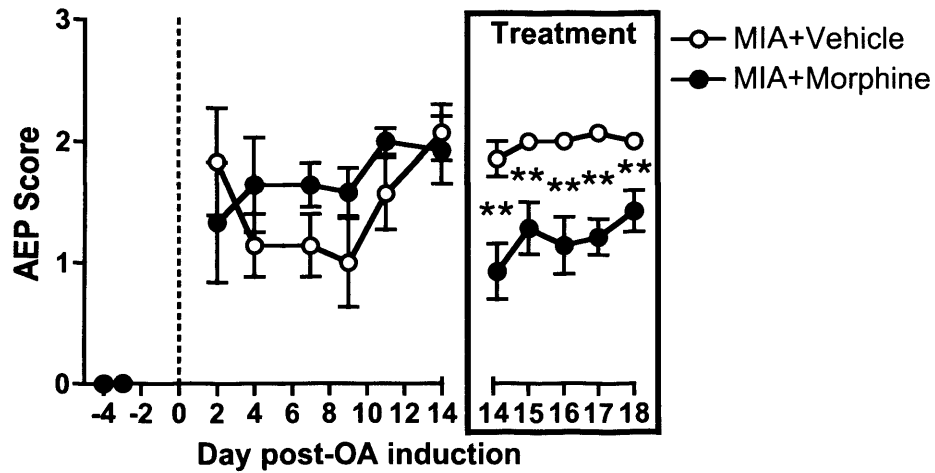
Figure 3.14. Effects of chronic (A) morphine 3 mg/kg or (B) gabapentin 30 mg/kg twice daily on cooling hypersensitivity, 30' post-treatment. A drop of acetone was applied a total of 5 times over the plantar surface of each hindpaw -ipsilateral (IL) and contralateral (CL) to the MIA injected knee. Data presented as mean  $\pm$  SEM,  $n=7$  in each group, comparisons using Mann-Whitney U-test. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ .

### 3.3.3.3 Ambulatory evoked pain score

Chronic morphine administration constantly decreased the pain score from the time of the first dose of day 14. This difference between the two groups was maximal on day 14, and was maintained until the last day of the treatment ( $P<0.01$ ), although the effect was slightly less on day 18 (Figure 3.15.A).

Gabapentin (Figure 3.15.B) also produced a prolonged statistically significant decrease in the ambulatory pain score but this effect only started on day 15 ( $P<0.05$ ) and tended to gradually become larger and more significant towards the end of the experimental run on day 18 ( $P<0.01$ ).

A.



B.

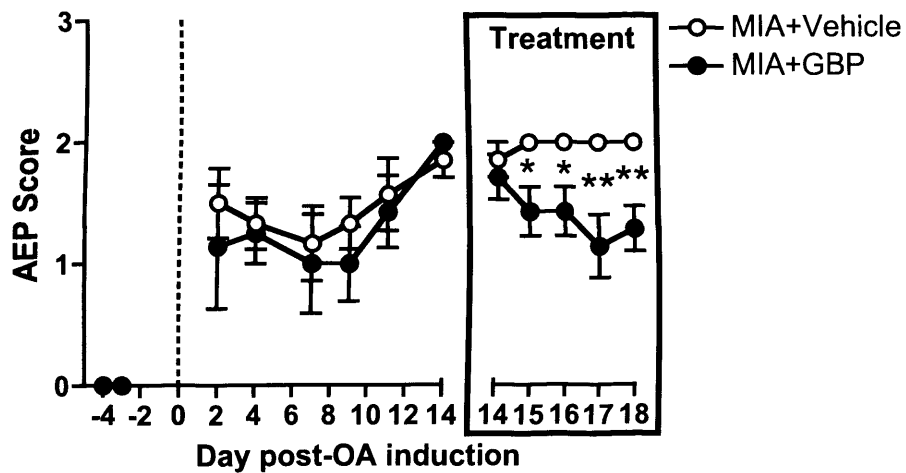


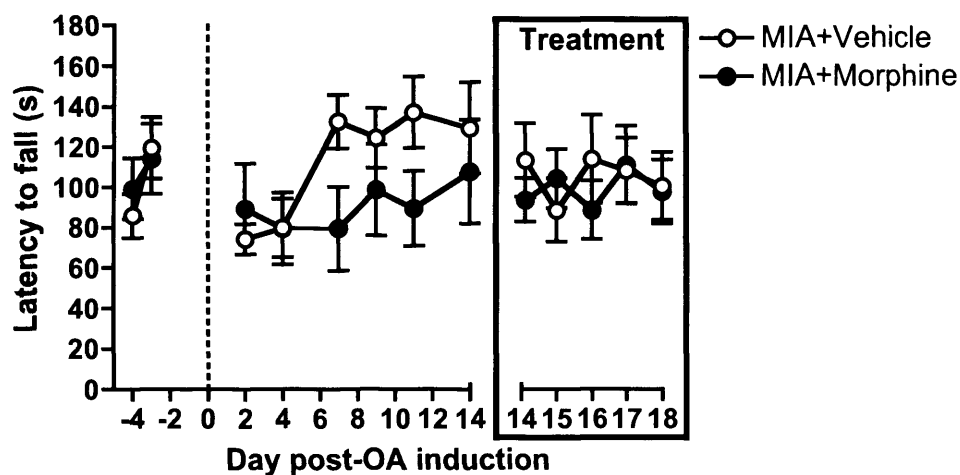
Figure 3.15. Effects of chronic (A) morphine 3 mg/kg or (B) gabapentin twice daily on the ambulation evoked pain score 30' post-treatment, using a rotarod. Data presented as mean  $\pm$  SEM,  $n=7$  in each group, comparisons using Mann-Whitney U-test. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ .

#### 3.3.3.4 Latency to fall

In the MIA animals, chronic morphine had no effect on the latency to fall when compared to controls, which indicates that at the dose of 3 mg/kg, there were no major side-effects effects in terms of sedation or motor impairment (Figure 3.16.A).

Chronic gabapentin (Figure 3.16.B), on the other hand had a slight tendency to decrease the latency to fall of actively treated animals, which proved to be maximal and statistically significant only on day 16 ( $p=0.0350$ ). Gabapentin could thus have had a slight sedative effect when administered chronically following that dosing regimen.

A.



B.

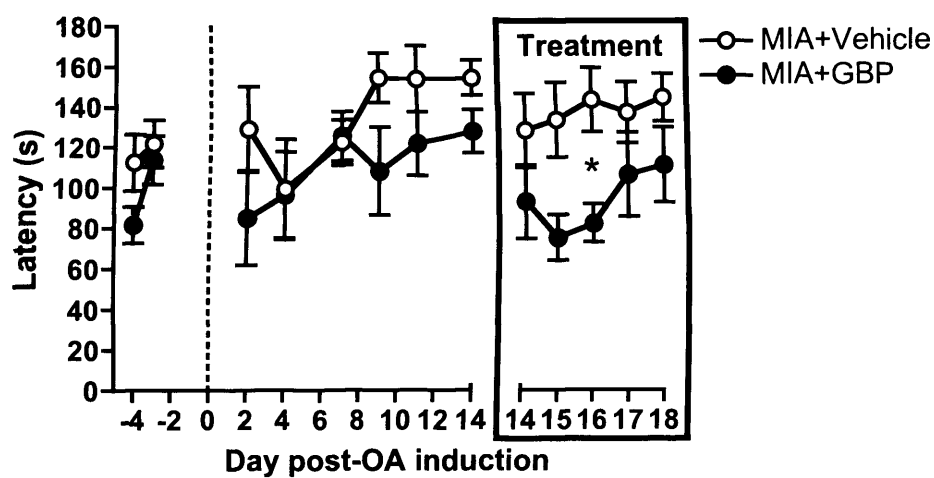


Figure 3.16. Effects of chronic (A) morphine 3 mg/kg or (B) gabapentin 30 mg/kg twice daily on the latency to fall, 30' post-treatment, assessed using a rotarod. Maximum allowed 180 s. Data presented as mean  $\pm$  SEM,  $n=7$  in each group, comparisons using Mann-Whitney U-test. \* $P \leq 0.05$ .

### 3.3.3.5 Latency to fall in sham non-arthritic rats

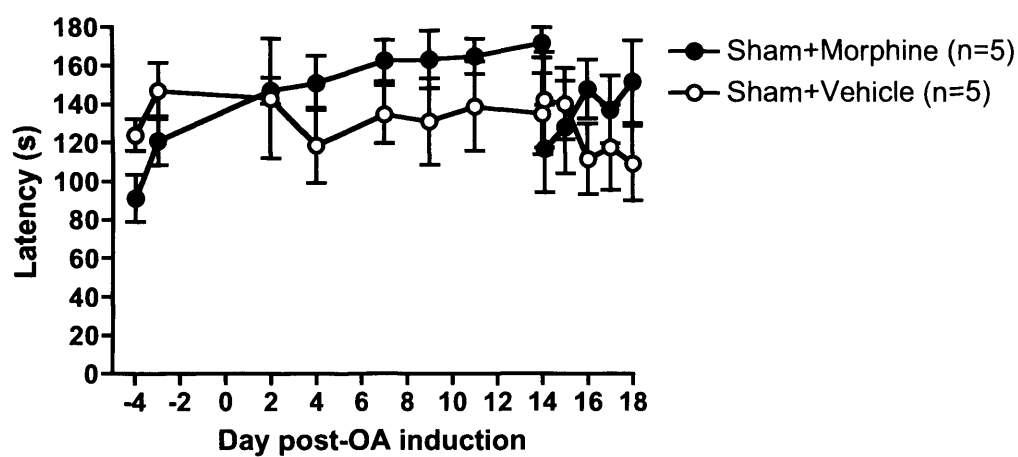
Both drugs were also tested on sham rats, in order to assess the potential side-effects such as sedation or motor impairment following their administration. While the mechanical and cooling sensitivity as well as the ambulation pain score were not affected, there were some changes for morphine and gabapentin on the rotarod.

Indeed, the chronic morphine treatment seemed to cause a slight trend towards a transient decrease in the rats' ability to stay on the rod (Figure 3.17.A), although that effect was very weak and at no time-point statistically significant.

Gabapentin chronic administration on the other hand created a rapid decrease in the rats' latency to fall, with statistically significant levels reached on days 15, 16 and 17 (Figure 3.17.B). However, this intensity of this effect seemed to decrease gradually.

It must be noted however that the general shape of graphs of responses for the control rats (before morphine or gabapentin treatment) were not exactly similar, because of the low number of animals in these groups ( $n=5$  and  $n=3$  for the control groups in the morphine and gabapentin experiments, respectively). In order to limit the number of animals used and because of the obvious effects of gabapentin, no additional animal was tested.

A.



B.

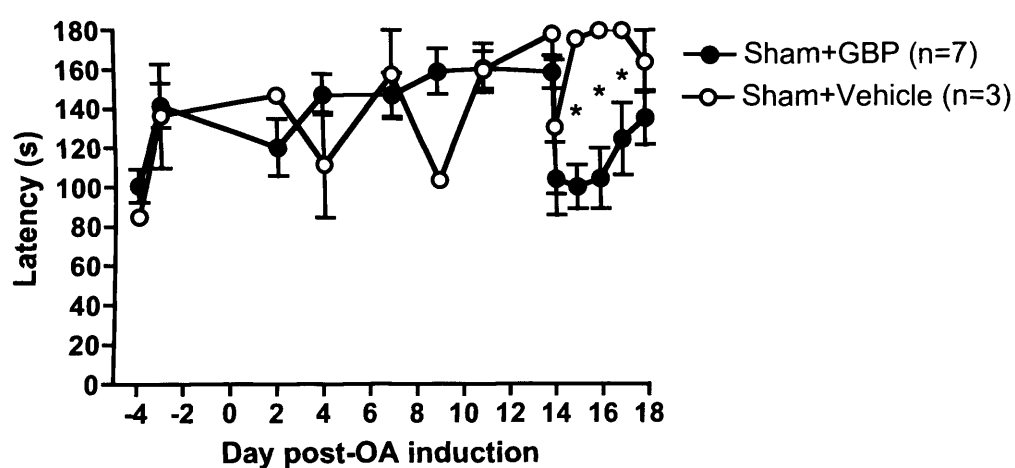


Figure 3.17. Effects of chronic (A) morphine 3 mg/kg or (B) gabapentin 30 mg/kg twice daily on the latency to fall, 30' post-treatment, in non-arthritic sham rats. Time limit was 180 s. Data presented as mean  $\pm$  SEM,  $n=7$  in each group, comparisons using Mann-Whitney U-test. \* $P \leq 0.05$

## 3.3.3.6 Weight gain

As shown on Figure 3.18, the animals gained weight normally during the experiments, which, again, suggested that neither the MIA model of OA pain, nor chronic morphine or gabapentin affected the animals' general health.

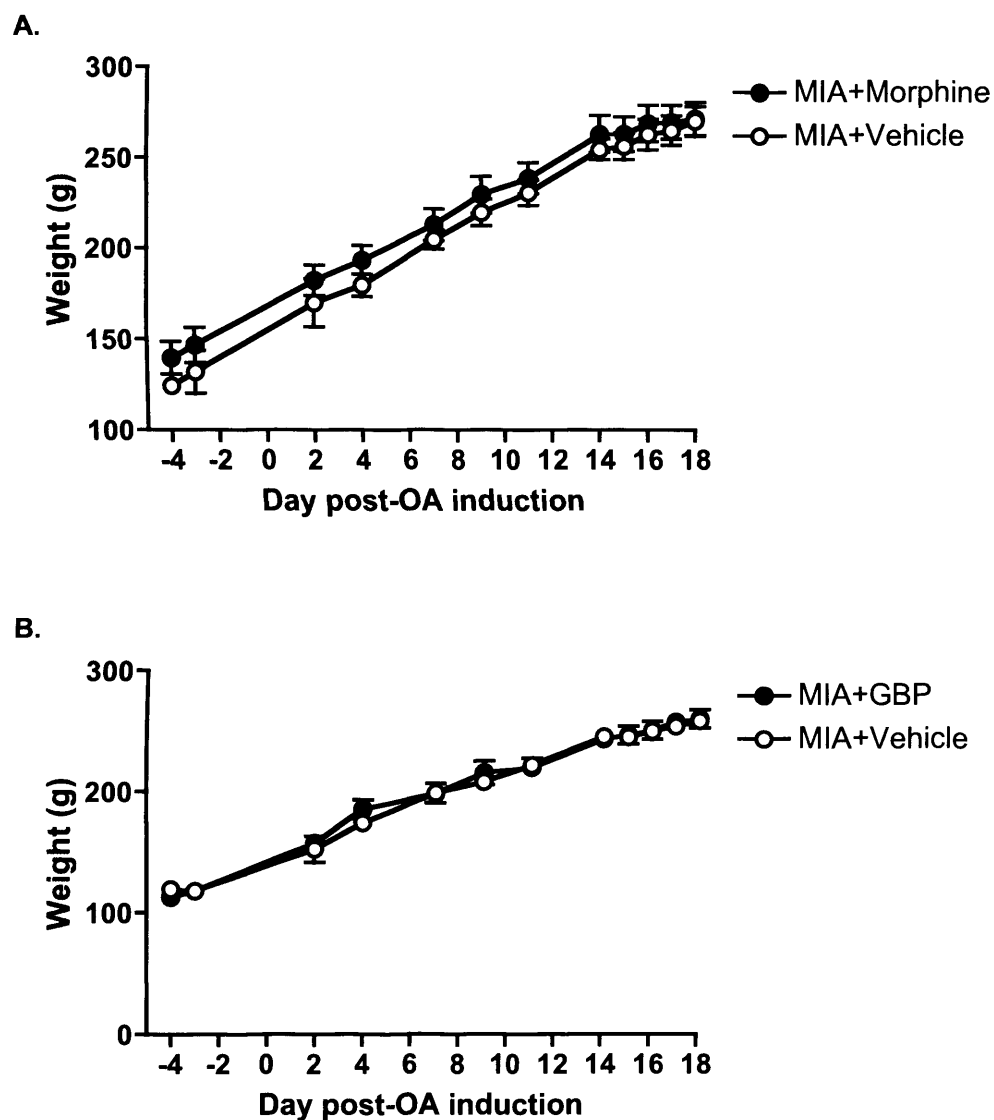


Figure 3.18. Weight gain during the development of the model and the chronic administrations of morphine (A) or gabapentin (B). Data presented as mean  $\pm$  SEM,  $n=7$  in each group.



## 3.4 Discussion

### 3.4.1 *Terminology/Nomenclature*

Contrary to some authors, and in agreement with Hansson and colleagues (A. Ekblom and P. Hansson, 1993, 1995), we should try to use the word 'pain' carefully in animal studies, and the term 'nociception' is often more appropriate, when referring to the IASP definition of pain. *A fortiori*, the word 'hypersensitivity' should be used in place of 'allodynia' and 'hyperalgesia', which can only be applied to human speaking subjects: indeed is it increased attention, hyperaesthesia, real frank pain (that would correspond to allodynia *per se*) and/or dysesthesia that contribute to mechanical hypersensitivity for example?

### 3.4.2 *Relevance and validity of the OA model to the human condition*

OA is a multifactorial disease where susceptibility (obesity, heredity, age...) and mechanical factors (a rupture of the anterior cruciate ligament ACL e.g.) are intimately related. OA pain is even more complicated as both the sensory and the affective components participate in pain perception. Indeed, emotional factors such as stress, depression or passive coping are proved to increase the pain ratings in such patients. As a result, it is easy to understand that this complex disease cannot be studied easily in humans, and that by studying animal models of OA, the contribution of the affective component of pain could be limited. However, as the aetiology of OA is very complex, it is easy to conceive that it cannot be modelled in a unique way, thus the existence of various types of OA models. The MIA model of OA pain in the rat has been used since 1987 (D. A. Kalbhen, 1987) and it has proved to give reliable and consistent results, in different groups all around the world and thus it has been established as a useful model to study OA pain (C. Guingamp et al., 1997; R. Combe et al., 2004; J. Fernihough et al., 2004; J. Fernihough et al., 2005; J. D. Pomonis et al., 2005; J. J. McDougall et al., 2006; S. P. Ivanavicius et al., 2007). My findings, using a dose of 2 mg MIA per animal for OA induction, are in agreement with these previous studies in terms of time-course and mechanical hypersensitivity, which supports the idea that the model is very robust for OA pain behaviour. Most of the other models seem to give more variable results, with an exception for the surgical ones (ACL transection e.g.), which however have a later onset for severe OA symptoms.

### 3.4.3 *Pain assessment and behavioural testing methods*

Some clinicians have asked why we stimulate the plantar surface and the toes whereas OA is induced in the joint: we tried to characterise this model of knee OA pain using new tools. Patients with generalised knee OA will describe their pain as troublesome, aching, hurting, nagging... and the pain could be elicited by various activities such as bending or walking (P. Creamer et al., 1998). Following a first systematic screening of virtually all the accessible joints of the body to identify abnormalities and establish their distribution and a more detailed analysis of an abnormal joint or group of joints, a basic orthopaedic examination would involve palpation of the knee for example, which could be informative on the localisation of the pain, abnormal sensations as well as inspection of the colour, skin or any other local changes, muscle wasting, swelling, deformity, temperature, tenderness, swelling of the bone, soft tissue or fluid... Flexion and extension would also be performed to check for movement-evoked pain and assess the range of movement.

To assess pain severity in OA patients, often the first motive of consultation, a major advance was the adoption of standardised, validated questionnaires such as the WOMAC (N. Bellamy et al., 1988), Lequesne, McGill Pain Questionnaire (MPQ) or a simple visual analogue scale (VAS). The WOMAC was recently shown to be more sensitive than other questionnaires, and did not appear to be influenced by anxiety and depression as much as the MPQ (P. Creamer et al., 1999).

In animals, the examination would be similar as you would observe the affected limb, try to ascertain how long the condition had been lasting for and how it had evolved. Then you would try to see if the limp is of increased or decreased weight-bearing, permanent or intermittent, at rest or after exercise, as well as the conditions of appearance of the limp: whether it followed a trauma or not, whether it was sudden or progressive or unknown. However, animals cannot talk about their sensations and pain in particular, which makes OA symptoms more difficult to assess.

In rats in particular, clues can only be gathered indirectly, for example by knee palpation, but this test can be a very stressful experience (and stress-induced analgesia is well described in rodents), in addition to the difficulty in assessment (vocalisation score) as well as the potential harm (biting) for the examiner! It was also shown that ultrasound vocalisation by rodents did not correlate with behavioural measures of persistent pain in any of the inflammatory (formalin test), visceral (referred hyperalgesia model following bladder inflammation) or neuropathic pain (partial spinal nerve ligation) models examined (V. C. Wallace et al., 2005). The gait changes have also been studied using a catwalk:

however the changes did not seem to be reversed by analgesics such as morphine in various models of chronic pain (unpublished data from Dr. F. Marchand, King's College London), which does not make this apparatus very useful to study novel drugs' effects. The analgesia could indeed be independent from the mechanical function for example. Other apparatus such as the incapitance tester could give complementary data, and it was used when testing a NSAID and a novel peripheral opioid receptor agonist, in Chapters 4 and 7 respectively.

These are some of the reasons why, in this rat model, I decided to study the mechanical and thermal sensitivity on the plantar surface and the toes of their hindpaws, after an adequate period of acclimatisation, and to use the rotarod to score their limp to have an idea of their ambulatory-evoked pain as well as to measure their latency to fall.

The use of a wide range of complementary techniques stimulating both the area of secondary hyperalgesia (with von Frey hairs and the acetone on the paw for example) and the area of primary hyperalgesia (using the rotarod - or the incapitance tester in Chapter 7) was hence here justified, in order to have a better picture of what is really happening in term of nociceptive behaviour.

It was interesting to note that whereas the von Frey and acetone tests gave biphasic responses associated with variable levels of differences between the sham and the OA groups, the rotarod score showed the development of a consistent and gradually increasing pain behaviour. The rotarod, by direct stimulation of the knee, thus correlates nicely with the development of a secondary hyperalgesia. It is clear that the different tests investigate different modalities of the nociceptive behaviour.

Moreover, the concept of testing the hindpaw (and not the knee itself directly) with von Frey hairs and acetone can be supported by the existence of referred pain. It is a feature of muscle pain that can be characterised by local pain and pain referred to distant somatic structures associated with cutaneous and deep somatosensory changes. This referred pain is dependent not only on central hyperexcitability but also on input from the periphery – the skin in particular (Laurson et al., 1997); moreover temporal and spatial summations are important on muscle experimental pain (Graven-Nielsen et al., 1997). Muscle hyperalgesia and an extension of the pain areas was also demonstrated when looking at nociceptive hypertonic saline injections into the *tibialis anterior* muscle in human OA patients, as a consequence of subsequent central sensitisation (P. Bajaj et al., 2001). All these elements supported the idea of studying the paw responses to evaluate OA pain, as long as the results were consistent and reproducible, which was the case here.

### 3.4.4 *Validation of the model*

#### 3.4.4.1 Mechanical hypersensitivity

When investigating the changes in mechanical hypersensitivity, my results using von Frey filaments testing are in agreement with previous paw withdrawal thresholds (PWT) studies by other groups (R. Combe et al., 2004; J. Fernihough et al., 2004) both in terms of threshold and time-course. The PWT determination consists of testing a broad range of von Frey hairs in ascending force order to determine the average paw withdrawal threshold. This technique, where the pressure is only applied to a single point of the paw, is very similar to the one I used, although I applied the same von Frey force filament 10 times over different areas of the paw and toes, which may be better as some areas of the paw could be more responsive than others.

I also found a biphasic phenomenon for the development of the mechanical hypersensitivity with von Frey 6 and 8 g, which is in agreement with the work by Bove et al. (2003) who showed that inflammation was only present during the first days after the injection and that by day 7, no apparent sign of synovial inflammation could be found. The proper osteoarthritic pain resulting from cartilage damage is likely to start to become predominant from that day onwards.

#### 3.4.4.2 Cooling hypersensitivity

The acetone drop test to assess cooling sensitivity has been used previously in a cancer-induced bone pain model (T. Donovan-Rodriguez et al., 2005). However, I measured for the first time the development of a clear cooling hypersensitivity in the MIA model from day 7, although days 11 and 14 were not statistically significant and only a tendency could be found on those days. However, during the pharmacological studies using morphine and gabapentin, this hypersensitivity was clearly diminished in the treated group in comparison to the vehicle group, except on day 16. This event on that day could have just been an experimental artefact, although these experiments were made in 4 different blinded and randomised batches of animals, and the other tests showed no similar feature (i.e. no anomalous average) on that day. It could also be a transitional stage for mechanisms responsible for the development of cooling hypersensitivity, which would then be attenuated again by both drugs.

#### 3.4.4.3 Ambulatory evoked pain score

The rotarod apparatus has been usually employed in order to assess motor function performance in 'normal' naïve rats or mice. It was then used to evaluate pain in a model of cancer pain in mice using a 'forced ambulatory guarding' rating, on a scale from 0 to 5 (N. M. Luger et al., 2002). Then it was used in another model of cancer-induced bone pain, looking at the 'ambulatory-evoked' pain score on a scale of 0 to 3 (T. Donovan-Rodriguez et al., 2004, 2005), which I decided to use. It is interesting to note that this rat scale was only from 0 to 3 whereas the mouse scale had 5 grades: this simplification of the score was made by Donovan-Rodriguez et al. (2004), as the scoring method itself was not easy and may have been more variable depending to the operator. By only having 3 grades instead of 5, the scoring method was then simplified and could be less variable. I also recorded the latency to fall, which was the only parameter entirely independent from human subjectivity. However, it did not show a large difference between the MIA and control groups and could not be used to quantitatively assess the pain-state. This may have been due to the fact that the pain was not severe enough and that because rats are four-footed, they could easily compensate their loss of motor function using their 3 other limbs, and no difference could thus be observed. This was in agreement with Donovan-Rodriguez's findings that unless rats had a limping score of 3, they would not have significantly different latencies to fall. This finding was also interesting as it was in agreement with the idea of having a mild model of pain and that the resulting induced pain state did not have a major impact on rats' general locomotory function, nor on their general health, as the weight curves did not differ between MIA and sham rats.

#### 3.4.4.4 Weight gain

It is interesting to note the lack of difference in terms of weight gain between the sham and OA groups, showing that the model used here is mild.

#### 3.4.5 *Drugs used and tested in OA treatment*

Pain is one of the few conditions in medicine where the numbers needed to treat (NNTs, number of people to be treated that will be necessary before a given level of efficacy is reached, for a given dose) are nearly always above 2, and are commonly around 3 or 4!

As shown in the introduction, a “ladder” was first developed by the WHO for cancer pain relief and aimed at simplifying pain relievers’ use and prescription. Basically, in case of cancer pain, people should be given drugs that are on the first step of the ladder: non-opioids such as aspirin and paracetamol that can be coupled with “adjuvants” i.e. additional drugs to calm fears and anxiety. If pain persisted or increased, there would be a move onto step two and mild opioids (codeine) would be added to the treatment. Finally, strong opioids such as morphine could be used, until the patient is free of pain.

These recommendations for cancer pain relief have been successfully applied to other pain conditions such as HIV/AIDS and can be applied to OA. However, as I already said, very few studies had looked at the effects of morphine, which justifies this work. Indeed there was only one study available and it showed that morphine reduced pain and improved several sleep measures versus placebo in moderate to severe OA patients (J. R. Caldwell et al., 2002). As for gabapentin, there were no studies available in humans.

#### 3.4.5.1 Morphine

My results recorded from the model on day 14 using mechanical stimulation, just after the first injection of morphine (3 mg/kg), were in agreement with other groups’ findings when they looked at acute morphine using similar doses. Combe et al. (2004) tested morphine at 0.3, 1 and 3 mg/kg on day 14, and showed an effect on punctuate hypersensitivity, as well as on weight-bearing, only using 1 and 3 mg/kg doses, in the same model. Fernighough et al. (2004) and Pomonis et al. (2004) found similar results. I also showed that acute morphine had a good effect at reducing cooling hypersensitivity and ambulatory evoked pain behaviour.

Concerning the use of chronic morphine, used in patients, no study had been done on the model. The administration of bi-daily morphine injections at the dose of 3 mg/kg and my results show that the opioid was very efficient and durably decreased mechanical and cooling hypersensitivity, as well as the ambulatory-evoked pain score, during the whole duration of the experiment with an immediate onset.

However some comments about the mechanical hypersensitivity results have to be made: morphine effects seemed to decrease towards the end of the treatment, which was particularly visible with von Frey 8 g results. This could be attributed to various factors such as the experimental factors and even tolerance. However, it could also be a “morphine-induced hyperalgesia”-like phenomenon caused by the treatment. This phenomenon was well known in humans and was described by clinicians in patients with increased pain sensations (See review by S. Arner et al., 1988). This opioid-induced

hyperalgesia was also shown in a murine model, following a treatment using morphine pellets (X. Li et al., 2001). Thus opioid tolerance could be the result of the development of a hyperalgesia counteracting the opioids effects, resulting in paradoxical pain behaviour that could offset the analgesia produced by the drug. A possible mechanism could be that neuroplastic changes occurring in the rostral ventral medulla, would activate descending facilitation pathways and increase the spinal expression of dynorphin, a peptide first identified as an endogenous kappa opioid agonist but that can be proprioceptive if highly expressed, and thus result in an abnormal pain state (T. W. Vanderah et al., 2001).

#### 3.4.5.2 Gabapentin

The other drug tested chronically is the newly marketed gabapentin, a voltage gated calcium channel alpha-2-delta subunit ligand, which is the only drug licensed to treat neuropathic pain. First used as anticonvulsant, then licensed for the treatment of neuropathic pain, gabapentin was shown to have an analgesic and opioid-sparing effect in acute postoperative pain management (K. Y. Ho et al., 2006).

Although no human clinical trial studying its effects on OA is available, it is believed that it could have a positive effect and some clinicians are using it in a few patients. In the MIA rat model of OA, Fernihough et al. (2004) tested acute gabapentin and only showed a small decrease of the PWT at day 14 and no reversal of hyperalgesia. Likewise, acute gabapentin had no significant effect on any of our tests on day 14. Chronic gabapentin, on the other hand, had a good effect at decreasing pain responses, in particular the ambulatory-evoked pain score. However, contrary to morphine whose onset of action was immediate after the first injection, gabapentin had a slower onset, with the best efficacy at the end of the 5-day treatment, when morphine effect was actually reduced in comparison to its effect at the beginning. This feature is also encountered in the clinical setting, as gabapentin needs to reach effective plasma and cerebrospinal concentration before any effect can be felt. It can certainly explain, at least partly, why Fernighough et al. (2004) hardly found any effect of acute gabapentin.

Besides those very interesting behavioural results concerning the use of gabapentin in OA-like conditions, I have to comment on the rotarod latency to fall, which was slightly altered by gabapentin but not by morphine, in both MIA and sham non arthritic rats. Gabapentin was used at the commonly used twice-daily dose of 30 mg/kg (T. Donovan-Rodriguez et al., 2005) for the duration of the treatment, which was not known to incur adverse effects on rat behaviour. Gabapentin was reported to have positive effects on anxiety, sleep and well-being in patients, which could explain, at least in part, that shorter

time spent on the rod. Higher acute doses of up to 100 mg/kg have already been used (J. Fernihough et al., 2004; S. P. Ivanavicius et al., 2007) in the MIA model of OA, as well in a surgical model of OA in Lewis rats (S. E. Bove et al., 2006), with no reported side-effects. Consequently, at the dose used in my study, gabapentin could have a mild sedative effect or an effect on motor coordination for example, although this effect seemed to be gradually reversed during the drug administration period. Indeed, this effect became non statistically significant from day 16-17. Its reversal could also be explained by a kind of learning or habituation behaviour. However, this effect on the latency to fall had no consequence whatsoever on the other modalities tested, as none of the time-course of the responses to the other tests was indicative of any sedative effects. All in all, the results suggest that the low sedative effects between day 14 and day 16, if any, were not responsible for the anti-nociceptive effects. After day 16, there was certainly no more sedation that could have influenced the pain responses. The rotarod is thus a useful tool when testing new drugs because of its higher sensitivity in detecting more subtle drug-induced side-effects.

The issue concerning the mechanisms of action of gabapentin on OA pain can be raised. As noted earlier, gabapentin works on voltage-gated calcium channels, by decreasing neurotransmitter release and action potential propagation in nociceptive neurones. It also has a central effect quoted earlier on well-being, sleep and anxiety. This combination of effects can result in decreased pain sensations. It was also suggested that gabapentin could play a role in peripheral sensitisation as it reduces the mechanosensitivity of fine afferent fibres in normal and inflamed rat knee joints (U. Hanesch et al., 2003). In this model of OA, it seems that gabapentin has more of a central effect as it takes a few days for the decrease in pain behaviour to occur, with no effect either on primary or secondary hyperalgesia at the beginning. Indeed, gabapentin has a half-life around 2 hours following oral and intravenous administration, with a maximum plasmatic peak following oral administration around the same time (L. L. Radulovic et al., 1995). Gabapentin is believed to readily cross the blood-brain barrier and to concentrate in brain tissue via an active transport process. However, this carrier-mediated transport mechanism of gabapentin through the blood-brain barrier system-L has a low  $K_m$ , making and is thus particularly susceptible to substrate saturation, especially at higher plasma concentrations (M. S. Luer et al., 1999). The chronic dosing regimen might thus have helped it to cross the blood-brain-barrier in order to produce its effects. Again, both primary (von Frey hairs and acetone) and secondary (ambulatory evoked pain score) hyperalgesia seemed to be decreased with gabapentin, with a more constant clean-cut effect on secondary than on primary hyperalgesia, which could be explained in part by the fact that gabapentin has the



ability to act both centrally and on some sensitised peripheral nociceptive fibres, in particular C-fibres, and thus reduce their mechanosensitivity.

In a dog surgical model of OA, PD-0200347, a gabapentin analogue, was also shown to reduce the expression and synthesis of MMT-13, a key mediator of OA (C. Boileau et al., 2005).

All these different mechanisms justify further investigation into the potential therapeutic use of gabapentin both in humans and animal models of OA.

### 3.4.5.3 Behavioural tests

It is interesting to note that the effects of both drugs were more consistent and clean-cut when looking at the ambulatory pain score using the rotarod than when studying mechanical and cooling hypersensitivity in the hind paws with von Frey hairs and acetone respectively. Indeed, the variability in the rotarod score results was smaller than with the other tests, which is in agreement with the fact that the rotarod investigates the effects of the drugs on the area of primary hyperalgesia i.e. the knee. Indeed pain evoked by movement in that area might well be more of higher intensity than the one in the area of secondary hyperalgesia i.e. the paws, as tested with von Frey hairs and acetone. Again, the usefulness of the different techniques is shown here, as well as the fact that the different tests are complementary and investigate different behavioural modalities.

I used several techniques stimulating both the area of secondary hyperalgesia (with von Frey hairs and the acetone on the paw for example) and the area of primary hyperalgesia (using the rotarod - and the incapacitance tester in Chapter 7) and both gabapentin and morphine reduced both types of measure of nociceptive behaviour. The effects of both drugs have been studied on secondary hyperalgesia induced by capsaicin in humans and were effective, suggesting a translational validity in my measures (J. Dirks et al., 2002; A. R. Frymoyer et al., 2007).

## **Chapter 4. Role of inflammation in the MIA model of OA**

## 4.1 Introduction

OA used to be thought of as a non-inflammatory joint disease, and was hence called osteoarthritis. It was then opposed to RA, which was the perfect example of a systemic inflammatory joint disease. However synovitis (or synovial inflammation) is a frequently observed phenomenon in osteoarthritic joints and contributes to the pathogenesis of OA through formation of various catabolic and pro-inflammatory mediators altering the balance of cartilage matrix degradation and repair (S. Sutton et al., 2007).

These inflammatory events are the visible consequence of a sporadic event. Inflammation is then thought to be present but only in peaks or waves, but not constantly.

It is well known that inflammatory cascades are produced and the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a well identified source of pain through sensitisation of primary afferents.

Following a noxious stimulus, inflammation can be triggered: from the hydrolysis of membrane phospholipids to the production of PGE<sub>2</sub>, several processes are involved.

From an enzymatic point of view, the main actors are PLA<sub>2</sub> and COX<sub>2</sub>. Both can be inhibited by glucocorticosteroids, while NSAIDs only target the COX enzymes.

On a neuromodulator point of view, the release of cytokines is a key element, as cytokines will enable the recruitment of leukocytes to the site of injury and the increased release of inflammatory mediators, which will contribute to exacerbate even more the pain sensation. TNF- $\alpha$  is one of those cytokines and some TNF- $\alpha$  antagonists exist.

In our MIA-induced model of OA pain, using immunohistochemical studies, inflammation was found during the first 7 days following MIA injection into the knee, and was found to be resolved by day 14, once the chronic OA-like nociceptive pain state is established (S. E. Bove et al., 2003).

### 4.1.1 *Aim of this study*

The aim of this study was to better understand the role of inflammation during the first 14 days following the induction of OA with MIA as well as afterwards, on the resulting long-lasting chronic pain state, by modulating it using different pharmacological agents at different time-points and by studying the subsequent pain behaviours.

My first plan was to try to block most inflammatory processes from the start, and thus limit the inflammatory effects produced by the MIA injection itself by co-administering it with one of the most potent anti-inflammatory drugs, an intra-articular glucocorticoid. How long would the glucocorticoid decrease the pain behaviours for? Would the so-called chronic nociceptive pain state post day 14 be affected by the blockade of inflammation from the start?

Due to the relatively long duration of action of glucocorticoids (in terms of weeks when administered intraarticularly), the second plan was to use a TNF-alpha antagonist that has a shorter duration of action, and give it during either the first or the second week following the MIA injection. As a result, we would then have a more specific idea about when inflammation is playing a key role in pain behaviour during those 14 days before the reliable chronic pain state was established.

Finally, my third plan was to study the effects of a NSAID with a good COX-2 specificity from day 14, which would give us an idea of its efficacy on the OA-like chronic pain state induced in this OA model.

#### **4.1.2 *Choice of the drugs used***

##### **4.1.2.1 Glucocorticosteroid**

Corticosteroids, have been used in both human and veterinary medicine for a long time, for example as emergency anti-inflammatory drugs in case of oedema of the glottis or anaphylactic shock.

Different molecules are available and were detailed in Table 1.5. (page 63).

Betamethasone had been used systemically in a rat model of collagen-induced arthritis (E. Larsson et al., 2004), but not only was it anti-inflammatory, immunosuppressive and with a strong mineralocorticoid effect, it also had a strong inhibitory action on the ACTH axis and a long duration of action, which did not make it the drug of choice for our study.

Methylprednisolone on the other had less mineralocorticoid effect as well as a shorter duration of action.

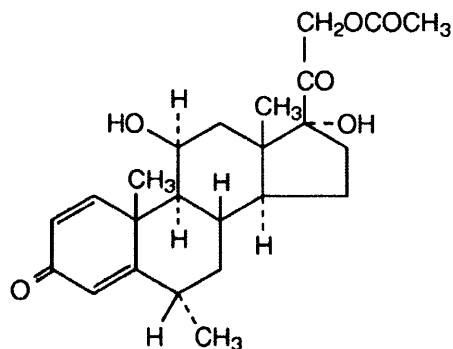


Figure 4.1. Structure of methylprednisolone acetate

The half-lives given in Table 1.6 follow oral administration. To avoid the systemic effect and to mimic the treatment already used in some patients, the intraarticular route was used. Indeed, the action of the glucocorticoid drug could then be restricted to the knee capsule and it could be slowly cleared from the synovial fluid. As the histology showed visible signs of inflammation within the synovium and surrounding tissues for one week only after the MIA injection (S. E. Bove et al., 2003), I decided to use methylprednisolone acetate (Depo-medrone<sup>TM</sup>, Pharmacia) and to co-administer it with MIA, by mixing the two together before giving a single intraarticular injection on day 0. Depo-Medrone<sup>TM</sup> (human drug) was preferred over Depo-Medrol<sup>TM</sup> (veterinary specialty), because the latter was only available as 5 ml of 40 mg/ml Methylprednisolone acetate whereas Depo-Medrone<sup>TM</sup>, the human drug existed in 1-ml vials of 40 mg/ml. They were otherwise identical in terms of composition.

According to the BNF, the indicated dose for the treatment of RA or OA in humans by intra-articular injection is 20-80 mg for a large joint (knee, ankle, shoulder) and 4-10 mg per small joint. In dogs, the average initial dose recommended for a large synovial space is 20 mg while smaller spaces would require a correspondingly lesser dose.

The dose for rats weighing about 150 g was extrapolated to 0.5 mg to be injected in the knee joint.

#### 4.1.2.2 TNF-alpha antagonist

Different TNF-alpha antagonists were available: monoclonal antibodies such as infliximab (Remicade<sup>TM</sup>) or adalimumab (Humira<sup>TM</sup>), or circulating receptor fusion proteins such as etanercept (Enbrel<sup>TM</sup>). They both had to be administered systemically.

Etanercept, a soluble TNF-alpha receptor widely used in RA conditions, was chosen as it had been used in a recent study showing the importance of TNF-alpha in the development

of inflammatory hyperalgesia in the CFA-induced arthritis model (J. J. Inglis et al., 2005). Indeed, TNF could play its role through two mechanisms: a direct action on neurones through TNFR1, as well as on macrophage accumulation in the DRG via a TNFR2-mediated pathway. The same dosing regimen used in that study was followed, i.e. 0.5 mg/kg in s.c. injections on alternate days, in this case either on days -1, 2 and 4, or on days 7, 9 and 11.

#### 4.1.2.3 NSAIDs: COX-1/COX-2 inhibitors

The mechanisms of action of NSAIDs have previously been discussed, but in short they are centred on the inhibition of COX enzymes. COX-1 is the constitutive isoform, while COX-2 is the inducible one following a noxious stimulus for example.

I wanted to administer a NSAID - with a preferential activity on COX-2 - on a chronic regimen, for 5 days from day 14 post-OA induction, to see its effects on the late chronic nociceptive pain, which would then be mimic a treatment administered in the clinics, either in humans or animals. I decided to use meloxicam.

Meloxicam, a NSAID of the oxicam class, is indeed used both in humans and small animals under the trademarks Mobic™ and Metacam™ (Boehringer Ingelheim) respectively.

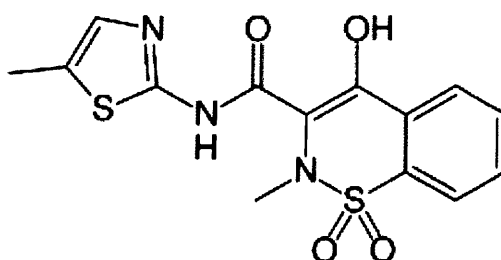


Figure 4.2. Structure of meloxicam (4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide) a novel NSAID of the acidic enolcarboxamide class.

Metacam™ is licensed for:

- Alleviation of inflammation and pain in acute and chronic musculo-skeletal disorders, such as osteoarthritis, in dogs.

- Reduction of post-operative pain following orthopaedic and soft tissue surgery in dogs; and after ovariohysterectomy and soft tissue surgery in cats.

Interestingly, because of those indications, Metacam<sup>TM</sup> is one of the most widely used NSAIDs in small animals and the most popular medicine used by veterinary surgeons in the UK to treat arthritis in dogs (Boehringer Ingelheim UK website).

Mobic<sup>TM</sup> is indicated in the treatment of pain and inflammation in rheumatic disease, exacerbation of OA (short-term) and ankylosing spondylitis in human patients.

Studies of meloxicam treatment of animals with adjuvant arthritis (a model of RA) revealed marked amelioration of the symptoms of bone and cartilage destruction and the systemic signs of immunologically induced inflammation (G. Engelhardt et al., 1995).

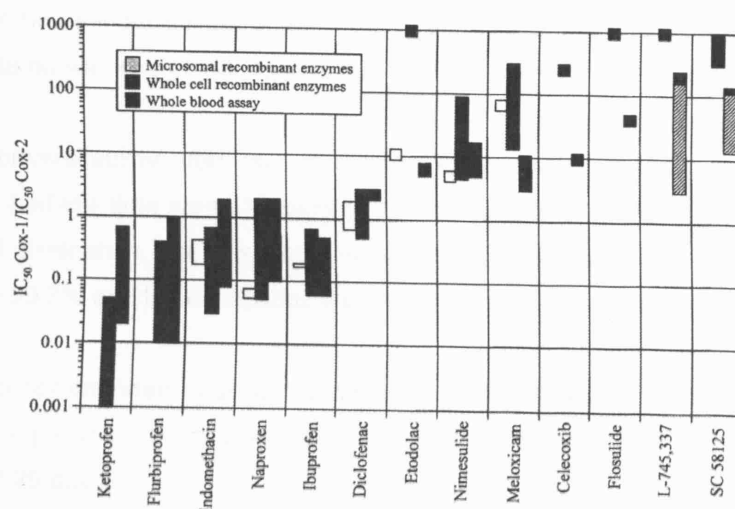


Figure 4.3. Selectivity ratios obtained for multiple NSAIDs using human microsomal recombinant assay (light grey bars), whole cell recombinant enzymes assay (dark grey bars) or human whole blood assay (black bars). Exact ratios could not be determined because IC<sub>50</sub> values for COX-1 inhibition were not obtained due to solubility problems at high concentrations (M. Pairet and J. van Ryn, 1998).

Meloxicam as can be seen from the figure above has good selectivity towards COX-2 inhibition, with high selectivity ratios COX-1/COX-2 in the range of [3-300] depending on the assay used (M. Pairet and J. van Ryn, 1998).

The great advantage of using meloxicam was that its pharmacological profile was very similar in both humans and rats, which could lead to a more reliable clinical translation from studies in the rodent species (U. Busch et al., 1998).

In rats, outside its licensed indications, Metacam<sup>TM</sup> was also reported to be well tolerated and was widely used in the treatment of post-operative pain.

These were the main reasons for the choice of meloxicam, although it was not a COX-2 specific NSAID *sensu stricto*.

In dogs, following oral administration, it has been shown that bioavailability was 100% with a plasmatic peak at 5-7 hours post-administration. After s.c. administration, the peak plasma level is reached at 2.5 hours post administration. Binding to plasma proteins was high (97%) and distribution volume was 0.3 l/kg. Half-life of meloxicam was found to be long, about 24 hours independent of the route of administration. Finally, around 75% of the dose administered was excreted in faeces and 25% by urine, through inactive metabolites.

In rats, the bioavailability after oral absorption is around 95%. Following a single i.v. injection, the half-life time was 13 hours in males, 37 hours in females resulting from a slower rate of elimination. There is also a very high protein binding after oral administration between 99.5-99.7% and the distribution volume was 0.1 l/kg (U. Busch et al., 1998).

In dogs, the recommended dose is 0.2 mg/kg (first dose) then 0.1 mg/kg (maintenance dose) with an equilibrium being reached in 3-5 days, associated with a maximum duration of treatment of 28 days.

The advised dose is 1 mg/kg once daily in rats, between 7.5 and 15 mg/day in humans.



## 4.2 Methods

### 4.2.1 *Intraarticular glucocorticoid treatment*

Following baseline testing (von Frey hairs 1, 6 and 8 g, acetone drop test and rotarod) on day -3, methylprednisolone acetate (Depo-medrone™, 40 mg/ml, Pharmacia Limited) at the dose of 0.5 mg/animal and MIA (2 mg/animal) were mixed in sterile saline to obtain a final volume of 25 µl. After homogenisation, it was injected in the right knee capsule using a 27G needle as for the normal MIA injections described previously. Control animals received MIA only. The day of the injection was referred to as Day 0.

Animals then underwent behavioural tests for 14 days post-injection on days 2, 4, 7, 9, 11, 14, 16 and 18.

### 4.2.2 *Anti-TNF-alpha therapy*

Etanercept (Enbrel™, 25 mg, Wyeth Pharmaceuticals,) was administered in the morning, either on days -1, 2 and 4 or on days 7, 9 and 11 post-OA induction, by s.c. injections in the scruff of the neck at the dosage of 0.5 mg/kg. Behavioural testing was done until day 14 and would normally start 30 minutes post-injection when etanercept was to be administered on a behavioural testing day.

### 4.2.3 *NSAIDs: meloxicam treatment*

Meloxicam (Metacam™ injectable solution, Boehringer Ingelheim Ltd) was administered once daily, in the morning, by s.c. injection with a 27 G needle at the dosage of 1 mg/kg (following dilution of Metacam™ in sterile saline, with a final concentration of 1 mg/ml) in the scruff of the neck, from day 14 until day 18 post-OA induction.

Behavioural testing was done on various days before the start of meloxicam treatment and daily, 30 minutes after the injection, when meloxicam was administered. On day 14, animals were first tested without any treatment in order to get a baseline response. The responses 30 minutes post-meloxicam on that day were plotted as day 14.5 on the graphs.

#### 4.2.4 *Behavioural tests*

The behavioural tests were realised as described in the main Methods section (Chapter 2). Von Frey hairs 1, 6 and 8 g were used to assess mechanical hypersensitivity, the acetone drop test to assess cooling hypersensitivity, the rotarod to assess both the ambulatory evoked pain score and the latency to fall. The incapacitance tester was also used to assess changes in hindpaws weight-bearing distribution for the meloxicam study only.

### 4.3 **Results**

I wanted to compare the effects of the different drugs for each behavioural test, i.e. mechanical sensitivity, cooling sensitivity, latency to fall, ambulatory evoked pain score as well as for meloxicam only, the changes in weight-bearing distribution.

#### 4.3.1 *Mechanical sensitivity*

Mechanical sensitivity was assessed using von Frey hairs with different bending forces.

##### 4.3.1.1 Von Frey 1 g

Von Frey 1 g results are shown on Figure 4.4. No abnormal hypersensitivity was noted. There was no difference at all between the control and the different drug-treated groups either.

##### 4.3.1.2 Von Frey 6 g

The results are shown on Figure 4.5.

Methylprednisolone (A) injected intraarticularly on day 0 had a major effect on mechanical sensitivity, on day 4 and 7 post-OA induction. It can also be noted that there was no difference on day 2. However this could have been caused by the low response frequency in the control animals (<3, normally between 5-7 for the other drugs): even though the methylprednisolone-treated group responses were even lower (<2) there was no statistically significant difference between the two groups.

Etanercept (B) administered early (on days -1, 2 and 4) produced an effect but only on day 4.

Etanercept (C) administered later (on days on 7, 9 and 11) did not provoke any changes. Finally, meloxicam (D) given daily between day 14 and day 18 only produced a statistically significant decrease in the response frequencies on day 18. However, the unexpectedly high level of responsiveness in the control group on that day may have caused this sudden change in the response trend.

#### 4.3.1.3 Von Frey 8 g

The results are shown in Figure 4.6.

Methylprednisolone co-administration with MIA (A) decreased the responses elicited by von Frey hair 8 g on days 2, 4, 9, which followed the same trend as von Frey 6 g results.

Early etanercept (B) caused a difference between the two groups tested with von Frey hair 8 g but only on day 4 post-OA induction, as with von Frey hair 6 g.

Late etanercept (C) did not have any effect.

Chronic meloxicam (D) had a reducing effect on those mechanical responses but only on day 15, i.e. on the second day of the chronic NSAID treatment.

#### 4.3.1.4 Conclusion for mechanical sensitivity

Generally speaking, the biggest and most obvious changes in mechanical hypersensitivity were seen with both von Frey hairs 6 g and 8 g, with even greater differences when using von Frey hair 8 g.

Treatment-wise, methylprednisolone injected at the same time than MIA had the longest lasting effect at reducing nociceptive behaviour, up to 11 days post-OA induction.

Etanercept also had an effect but only when administered early, and this was limited to day 4, i.e. to the third and last day of etanercept administration (given on days -1, 2 and 4).

Late etanercept, administered on day 7, 9 and 11, had no effect on mechanical sensitivity.

Finally, the administration of meloxicam between days 14 and 18, i.e. on the late chronic nociceptive phase, gave mixed results: whilst a clear trend towards a reduction in mechanical sensitivity was seen during that treatment period, statistically significant differences were only reached sporadically.

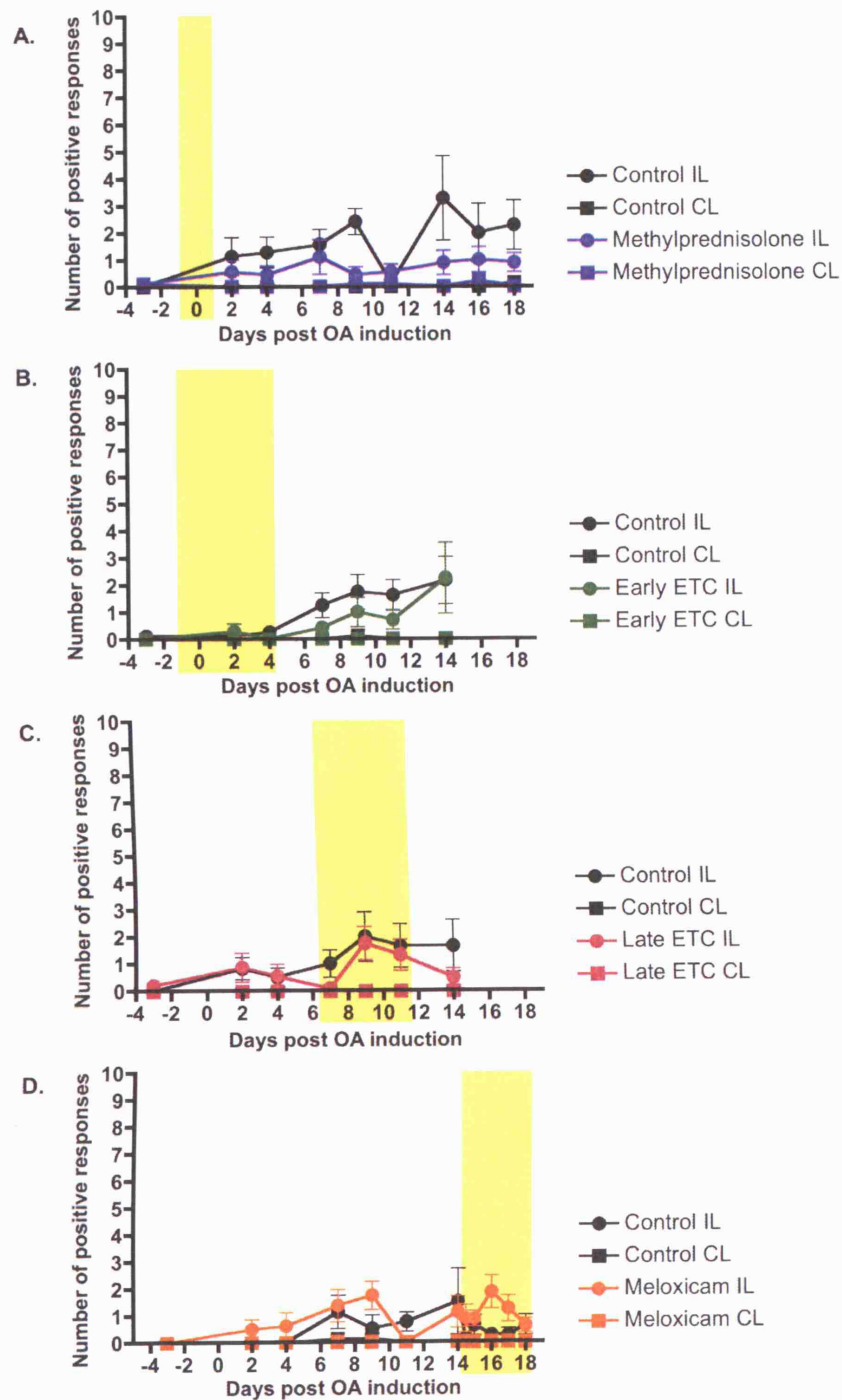


Figure 4.4. Mechanical testing of the hindpaws using von Frey hair 1 g, with the administration of (A) methylprednisolone intra-articularly on day 0 ( $n=9$ , controls  $n=7$ ), (B) etanercept s.c. on days -1, 2 & 4 ( $n=7$ , controls  $n=8$ ), (C) etanercept s.c. on days 7, 9 & 11 ( $n=9$ , controls  $n=6$ ), or (D) meloxicam s.c. on days daily between days 14 and 18 ( $n=8$  in each group). IL: ipsilateral to the lesioned limb, CL: contralateral. Statistical analysis using Mann-Whitney test, \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ .

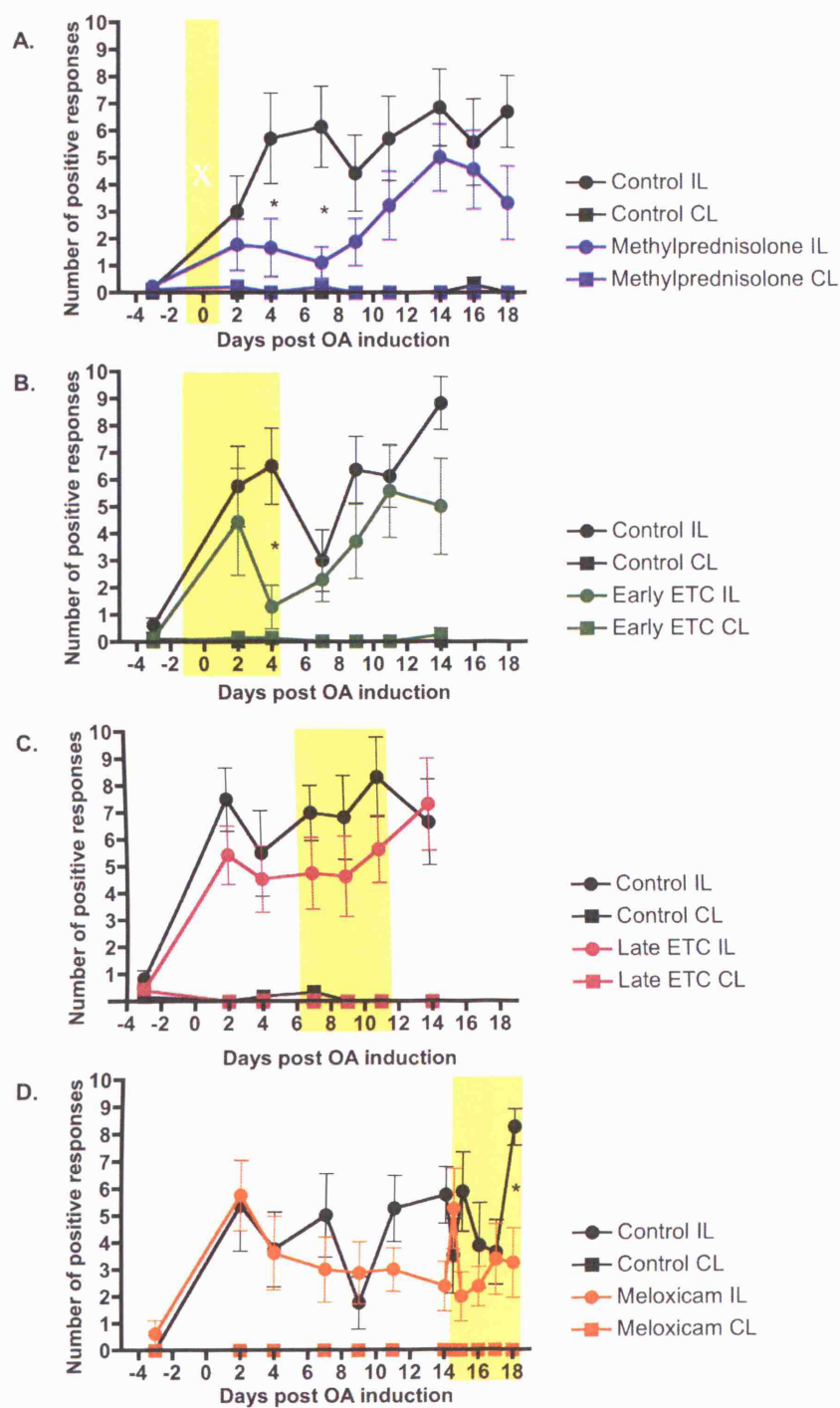


Figure 4.5. Mechanical testing of the hindpaws using von Frey hair 6 g, with the administration of (A) methylprednisolone intra-articularly on day 0 ( $n=9$ , controls  $n=7$ ), (B) etanercept s.c. on days -1, 2 & 4 ( $n=7$ , controls  $n=8$ ), (C) etanercept s.c. on days 7, 9 & 11 ( $n=9$ , controls  $n=6$ ), or (D) meloxicam s.c. on days daily between days 14 and 18 ( $n=8$  in each group). IL: ipsilateral to the lesioned limb, CL: contralateral. Statistical analysis using Mann-Whitney test, \*  $P \leq 0.05$ .

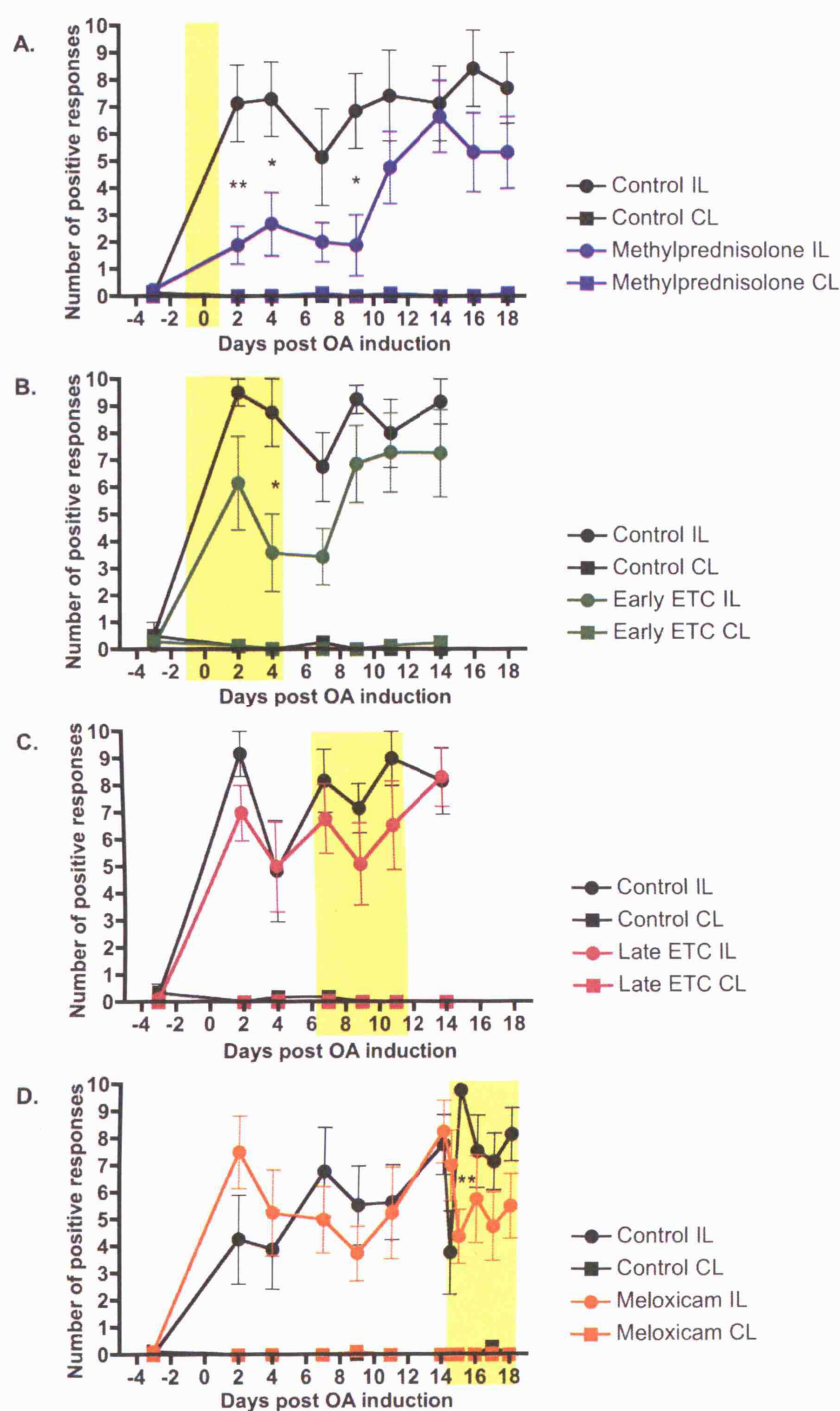


Figure 4.6. Mechanical testing of the hindpaws using von Frey hair 8 g, with the administration of (A) methylprednisolone intra-articularly on day 0 ( $n=9$ , controls  $n=7$ ), (B) etanercept s.c. on days -1, 2 & 4 ( $n=7$ , controls  $n=8$ ), (C) etanercept s.c. on days 7, 9 & 11 ( $n=9$ , controls  $n=6$ ), or (D) meloxicam s.c. on days daily between days 14 and 18 ( $n=8$  in each group). IL: ipsilateral to the lesioned limb, CL: contralateral. Statistical analysis using Mann-Whitney test, \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ .

### 4.3.2 *Cooling sensitivity*

The cooling sensitivity results are presented on Figure 4.7.

Methylprednisolone treatment (A) induced a clear trend towards lower response frequencies to acetone between days 4 and 18, but no statistically significant differences.

Early etanercept (B) treatment resulted in a slight trend towards inhibition of cooling responses, from day 2 to day 18.

Late etanercept (C) caused a statistically significant difference in the acetone responses on day 7, with lower responses for the treated group. However, lower responses in this group, were visible from day 2 until day 14, i.e. even before the etanercept treatment started, although the differences were not statistically significant.

Chronic meloxicam (D) had no effect on the cooling sensitivity at any time-point.

In fact, none of the drugs used in this study had a statistically significant effect on cooling hypersensitivity.

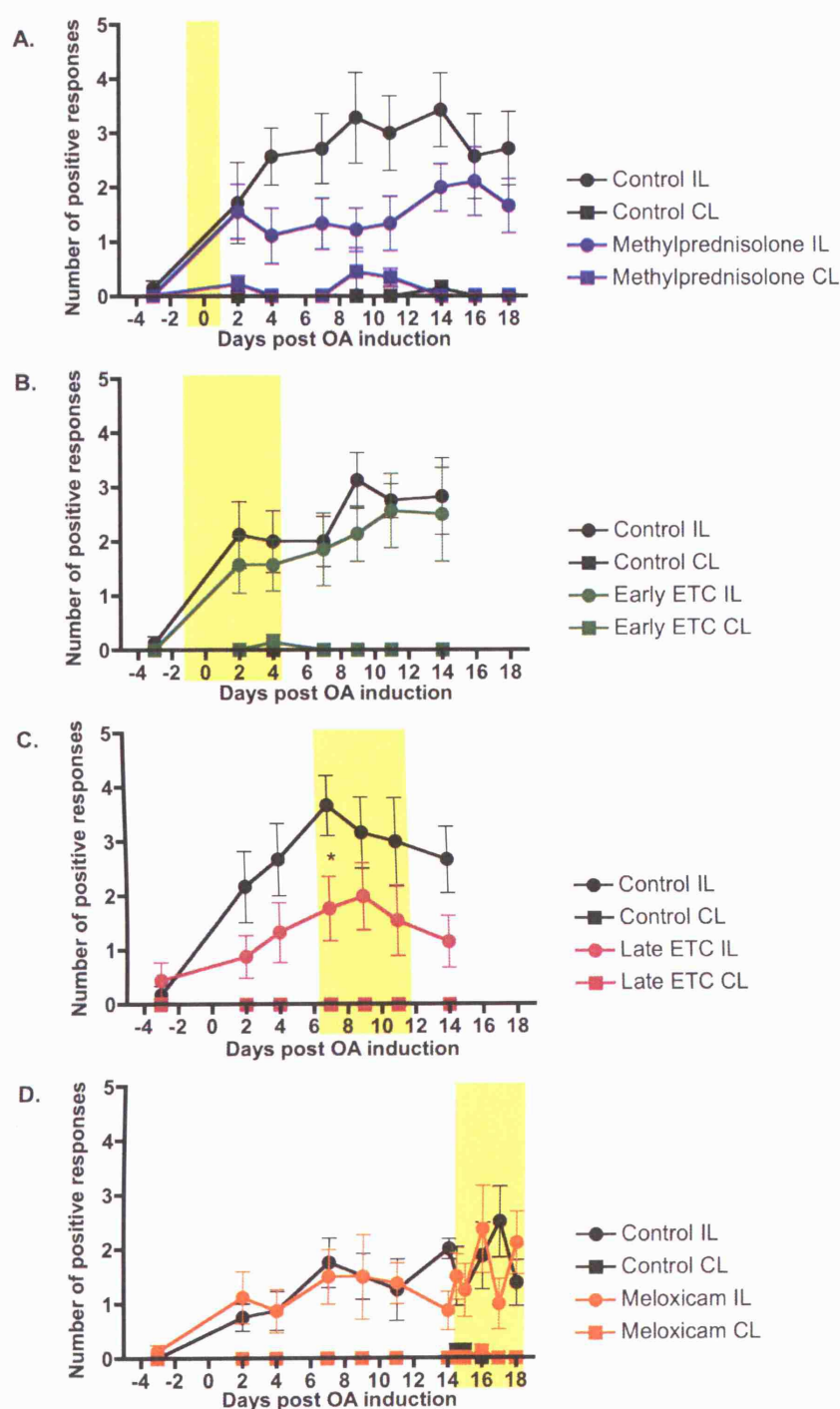


Figure 4.7. Cooling sensitivity assessment on the hindpaws using the acetone drop test, with the administration of (A) methylprednisolone intra-articularly on day 0 ( $n=9$ , controls  $n=7$ ), (B) etanercept s.c. on days -1, 2 & 4 ( $n=7$ , controls  $n=8$ ), (C) etanercept s.c. on days 7, 9 & 11 ( $n=9$ , controls  $n=6$ ), or (D) meloxicam s.c. on days daily between days 14 and 18 ( $n=8$  in each group). IL: ipsilateral to the lesioned limb, CL: contralateral. Statistical analysis using Mann-Whitney test, \*  $P \leq 0.05$ .



### 4.3.3 *Ambulatory evoked pain score (AEPS)*

The rotarod AEPS results are shown on Figure 4.8.

Straight from the start, methylprednisolone pre-treatment (A) resulted in decreased pain scores, with statistically significant differences on days 2, 4 and 9. Day 7 was normally a day when responses reached a minimum, so it was not surprising that responses were not statistically significant on that day. This trend lasted until day 11. From day 14 and until day 18, the difference between the control and the treated groups vanished, suggesting that the late chronic nociceptive phase is not so dependent on the primary inflammatory phase following OA induction with MIA.

Early etanercept treatment (B) did not have any effect except on day 7, i.e. 3 days after the last drug administration.

Late etanercept (C) had no effect at all on the pain score.

Meloxicam treatment (D) resulted in lower pain scores both acutely i.e. 30 minutes after the first treatment on day 14, and chronically on days 16 and 18. This suggests, based on the pain score, that the late chronic nociceptive pain state is somehow sensitive to meloxicam, although that effect is not consistent throughout the treatment period.

However, these reductions were smaller (in magnitude) than the ones seen between days 2 and 11 following methylprednisolone treatment (A).

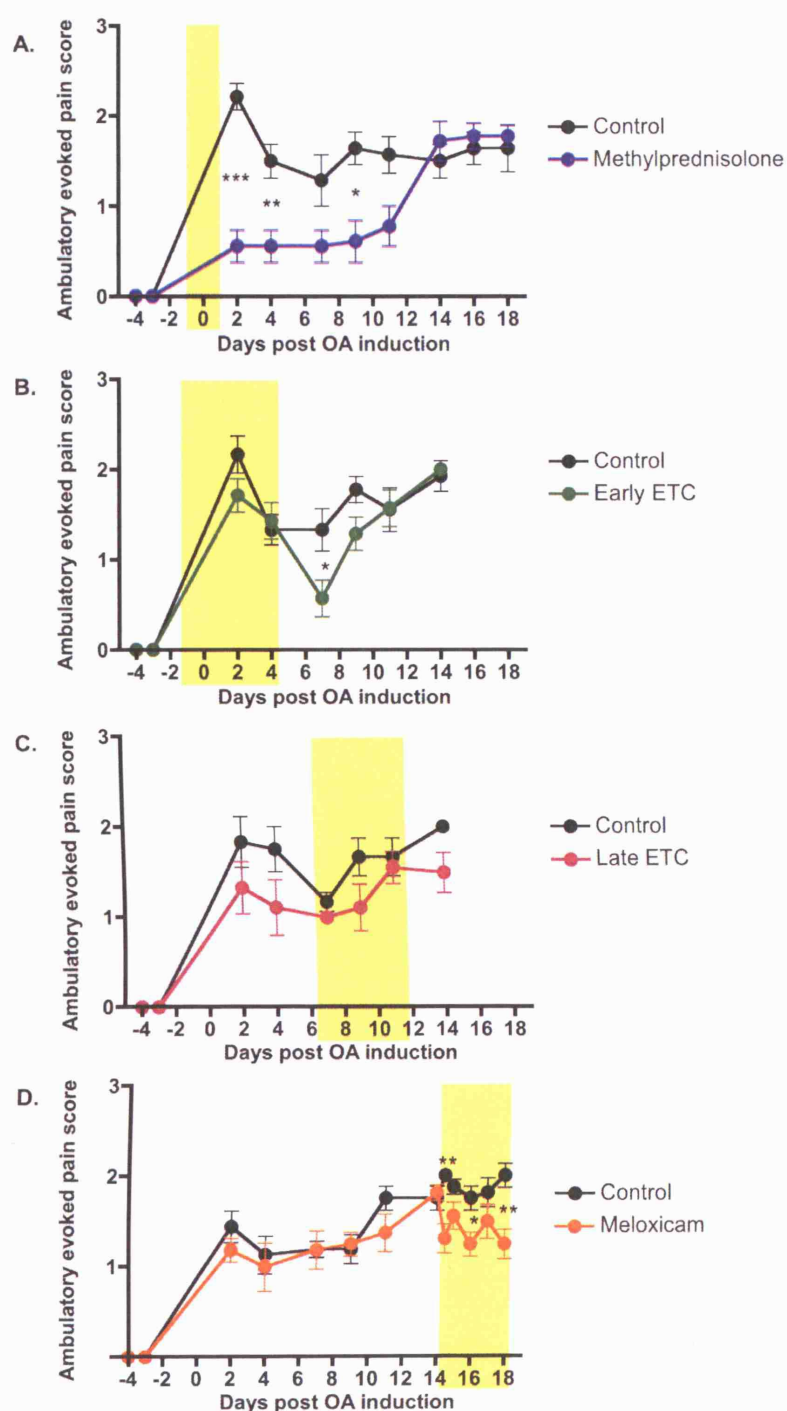


Figure 4.8. Assessment of ambulatory evoked pain score using the rotarod device, with the administration of (A) methylprednisolone intra-articularly on day 0 ( $n=9$ , controls  $n=7$ ), (B) etanercept s.c. on days -1, 2 & 4 ( $n=7$ , controls  $n=8$ ), (C) etanercept s.c. on days 7, 9 & 11 ( $n=9$ , controls  $n=6$ ), or (D) meloxicam s.c. on days daily between days 14 and 18 ( $n=8$  in each group). IL: ipsilateral to the lesioned limb, CL: contralateral. Statistical analysis using Mann-Whitney test, \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ .

#### 4.3.4 *Latency to fall*

The results are presented on Figure 4.9.

Methylprednisolone intra-articular administration (A) resulted in a trend towards an increased latency before falling off the rod at all tested time-points between day 2 and day 18.

Neither the early (B) nor the late (C) etanercept treatments had an influence on the latency to fall.

In the meloxicam experiment (D), it was interesting to note that the meloxicam to-be-treated group followed a trend towards poorer performance than the control group between days 2-14. However, on days 17 and 18, i.e. on days 4 and 5 of the chronic meloxicam treatment, this trend disappeared and both groups ended up reaching the same latency.

The latency to fall was not decreased by any of the treatments, which suggests that there was no locomotor impairment or sedation produced by the different drugs used, and which is in agreement with the pharmacological effects of those drugs.

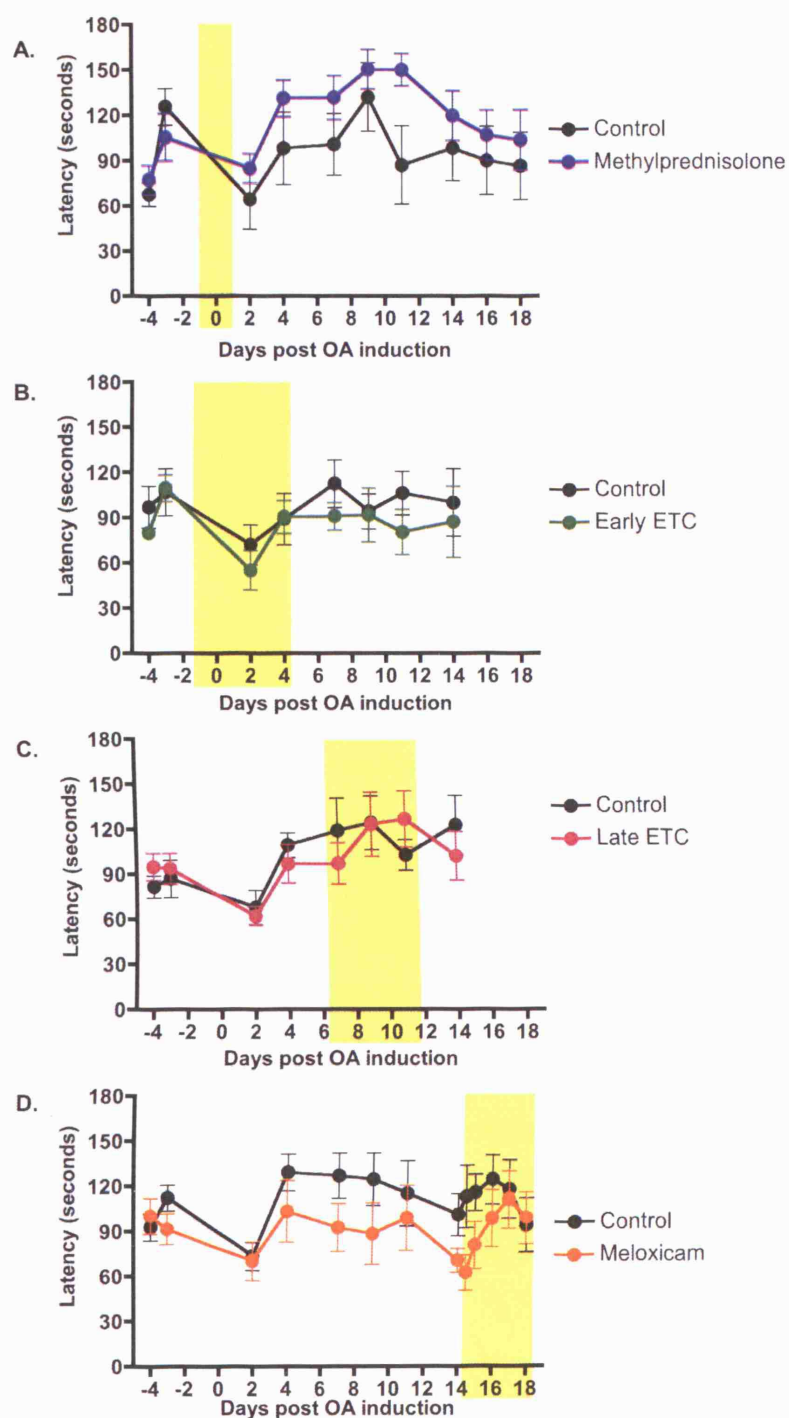


Figure 4.9. Assessment of the latency to fall using the rotarod device, with the administration of (A) methylprednisolone intra-articularly on day 0 ( $n=9$ , controls  $n=7$ ), (B) etanercept s.c. on days -1, 2 & 4 ( $n=7$ , controls  $n=8$ ), (C) etanercept s.c. on days 7, 9 & 11 ( $n=9$ , controls  $n=6$ ), or (D) meloxicam s.c. on days daily between days 14 and 18 ( $n=8$  in each group). IL: ipsilateral to the lesioned limb, CL: contralateral. Statistical analysis using Mann-Whitney test.

### 4.3.5 Weight-bearing

The incapacitance tester, which was not available until the meloxicam study was used to assess the weight-bearing changes produced by meloxicam.

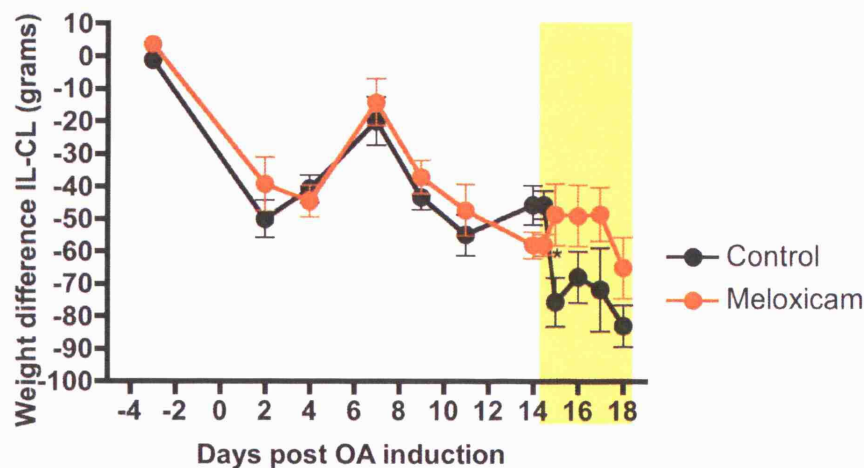


Figure 4.10. Assessment of weight-bearing changes between the ipsilateral and the contralateral hindpaws using an incapacitance tester, with the administration of meloxicam s.c. on days daily between days 14 and 18 ( $n=8$  in each group). IL: ipsilateral to the lesioned limb, CL: contralateral. Statistical analysis using Mann-Whitney test, \*  $P \leq 0.05$ .

Figure 4.10 shows that before the meloxicam treatment, from days -3 to 14, both the treated and control groups presented the same weight-bearing difference between their ipsi- and contralateral hindpaws. However, although there were no changes half an hour after the first dose of meloxicam on day 14, a statistically significant difference appeared on day 15, showing that the drug increased the weight borne on the ipsilateral lesioned side (difference of 27 g). This effect seemed to last until 18 as there was a clear trend towards the same effect, although there was no more statistically significant difference.

## 4.4 Discussion

### 4.4.1 *Role of inflammation in the induction of the MIA model of OA pain*

As expected, methylprednisolone mixed with MIA to induce OA had a great effect at reducing inflammation and the associated behaviour pain for a period of up to 11 days post administration. Indeed, inflammation had been previously studied and had been found to be present mainly during the first 3 days, before resolving by day 7 (S. E. Bove et al., 2003).

This long-lasting effect reproduced what was already known in both humans and animals, i.e. that the intra-articular injection of a glucocorticoid in an inflamed joint produced an immediate decrease of the inflammatory response as well as a reduction in the pain response, in this case for up to 11 days following OA induction, when inflammation was mainly found to be present during the first 7 days.

The very intriguing finding was that even though inflammation was blocked from the start of OA induction and the pain responses decreased until day 11, the late chronic nociceptive pain state was still present from day 14. The idea of this late OA chronic pain state being mainly due to nociceptive pain rather than inflammation was then highly reinforced.

It was also the first time this drug has ever been administered in rats' knee joints, and the 0.5 mg/animal dose of methylprednisolone acetate was therefore appropriate.

The use of the TNF-alpha inhibitor etanercept was to verify that the hypothesis that the pain responses recorded during the first week post-OA induction were more due to inflammation than those from the second week.

My findings indeed confirmed that etanercept, but to a much smaller extent than methylprednisolone, had a statistically significant effect on day 4 (following treatment on days -1, 2 and 4) on mechanical sensitivity, as well as on day 7 on the ambulatory evoked pain score, whereas it had no effect at all when administered during the second week, on days 7, 9 and 11.

In both cases, the effects of etanercept, if any, was very short-lived and like methylprednisolone, did not prevent the development of the chronic OA pain state from day 14. This confirmed that inflammation was not so critical on the induction of OA pain.

The role of inflammation during the first 14 days could then be a visible “side-effect” of OA induction using MIA. Indeed, the MIA solution used was quite concentrated (80 mg/ml) and it obviously had an immediate irritant and inflammatory effect, maybe due to its crystalline structure, based on the responses we could record on day 2 post-injection in comparison to the saline controls. Thus the MIA solution could first produce an inflammation due to the compound itself, and then produce its actions - that are slow to start - at the same time: first some synoviocytes for example, the cells that are producing the synovial fluid and that are in direct contact with the latter, but also a little later some chondrocytes will have their metabolism inhibited and will thus progressively degenerate. All these reactions will participate in the release of cellular, matrix and enzymatic debris, which in turn could initiate but mainly maintain an inflammatory pain state, until MIA has been cleared from the synovial fluid. At that time, the chronic nociceptive OA-like pain state then become predominant.

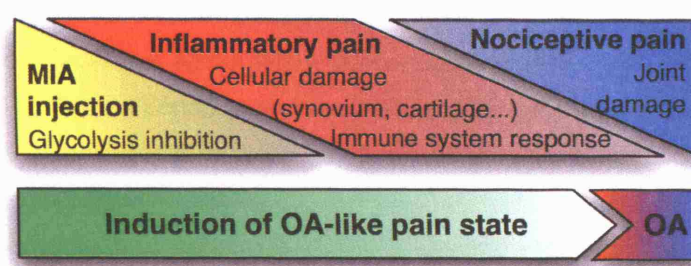


Figure 4.11. A hypothesis for the role of inflammation in the MIA model of OA pain. The injection of MIA would be accompanied by a primary inflammatory phase caused by the compound's physical structure and activity: the inhibition of glycolysis in cells from the synovium and as well as in the cartilage tissue for example would contribute to the production, in the synovial fluid, of various debris that would need to be cleared by some cells of the immune system. Chondrocyte death would gradually lead to cartilage damage, and whilst inflammation would be resolving, a chronic nociceptive pain state would become preponderant. The OA pain state would be a combination of both inflammatory (by peaks) and nociceptive pain.

Following this hypothesis, by blocking inflammation with methylprednisolone, the initial inflammatory response has been reduced, but the cartilage degenerative process through inhibition of glycolysis in chondrocytes still takes place, hence the presence of the OA-like pain state by day 14.

This does not explain why etanercept did not work well at all during either the first or the second week, as it is thought to be a potent anti-inflammatory compound because of its action in the early inflammatory response. It could be hypothesised that the death of some cells such as synoviocytes, as well as a few chondrocytes, would trigger the release of

much debris in the synovium, which will need to be cleared out by mechanisms such as phagocytosis. These debris, just by their presence, could have a direct mechanical irritant action on the afferents present in the knee joint as well as participate in the disruption of chondrocyte metabolism.

#### **4.4.2 *Role of inflammation on the chronic nociceptive pain state after day 14***

The use of meloxicam on the chronic nociceptive pain state revealed an effect that was not either of great amplitude nor very persistent. Indeed, with von Frey hair 8 g, although there was a trend towards a decrease in mechanical hypersensitivity, this effect was only statistically significant at one time-point, on day 15. The same tendency was observed with the incapacitance tester (statistical significance reached on day 15), as well as with the rotarod and the associated ambulation evoked pain score that was lower with meloxicam and statistically significant on three occasions during the treatment, on days 14, 16 and 18. These results were broadly in agreement with previous findings.

- Diclofenac (30 mg/kg s.c.) and paracetamol (300 mg/kg p.o.) were shown to reduce “pre-dose mechanical hyperalgesia” only on day 3 but at no other time-point, in particular not on day 14 (J. Fernihough et al., 2004).
- Acute indomethacin and celecoxib (COX-2 specific inhibitor) had no effect when administered acutely on day 14, but chronic celecoxib (30 mg/kg) given for 10 days had an effect on weight-bearing (J. D. Pomonis et al., 2005).
- Transient and weak effects were observed with naproxen (0.3-10 mg/kg) on days 14 and 28, whereas celecoxib (1-10 mg/kg) showed no significant effect (S. P. Ivanavicius et al., 2007).

However, it was also shown that paracetamol, naproxen (COX-1/2 inhibitor), rofecoxib (COX-2 specific inhibitor) at doses between 0.1 and 100 mg/kg worked well on day 14, at 2, 4 and 6 hours post-administration, after weight-bearing measurement (S. E. Bove et al., 2003). This last group, along with Bove et al., Pomonis et al. and Ivanavicius et al. injected 1 mg of MIA into the knee joint, whereas we followed Combe et al. and Fernihough et al. and used 2 mg. This might explain the results obtained by Bove et al.: a less severe condition could be more sensitive to NSAIDs but overall, all the results converged towards one fact: NSAIDs have a good efficacy when administered early following the induction of the model and are much less efficacious later.



These results could be explained by two hypotheses: either the dose of NSAID used, in our case meloxicam, was not high enough, or there was no or little inflammation.

The dose of meloxicam used in this study, 1 mg/kg once daily, is the generally accepted dose currently used in rats in animal units all over the world for the treatment of post-operative pain. Meloxicam has a greater effect on COX-2 rather than COX-1, producing a situation very similar to that seen in the clinics with human patients where –even at the right dose- the analgesic and anti-inflammatory activity of COX-2 specific inhibitors was somewhat inferior to other non-selective acidic compounds.

While the problem of dose escalation will be discussed later, the great variability and inconsistency in the responses of meloxicam-treated rats, on a day-to-day basis, especially to von Frey hair 8 g responses and the rotarod, especially the ambulation evoked pain score, would tend to suggest that the issue would be more the role of inflammation in this chronic pain state.

Indeed, as was discussed earlier, inflammation is most important (and causing pain) in the MIA model during the first two weeks, especially during the first seven days following OA induction. The total absence of inflammation from a histological point of view in the joint by day 14 (S. E. Bove et al., 2003), lead to the hypothesis that there was no inflammation thereafter, or at least that inflammation was playing a minor role in OA pain in this model. The variability of the responses during meloxicam treatment has thus been shown, although it is necessary to look back at the responses of the control groups: these were quite variable too, the best example being the averaged response to von Frey hair 6 g on day 18 in the control group in comparison to the meloxicam group. Indeed on that day, the control group underwent a huge increase in its response frequency, in comparison to the previous day, which made the difference with the meloxicam group statistically significant. Although this sudden increase in the control group on that day could have been considered artifactual, it could actually have mimicked very well what happened in real OA patients or animals. Indeed, we previously discussed the controversy some time ago when osteoarthritis was called osteoarthrosis – to enhance the non-inflammatory condition – which was later changed back to osteoarthritis, as inflammation, even if not constantly present, was thought to play a role in this rheumatic condition. Indeed, both after day 14 in our model and in OA patients, there could be sporadic peaks of inflammation: either short inflammatory episodes - or longer-lasting ones – in reaction to the release or simply the presence of freely moving osteo-cartilaginous fragments, which contribute to trigger and maintain inflammation.

Most of the time, the liberation of cartilaginous debris could cause little inflammation, which would resolve rapidly.

Another episode could return and last longer: in that case, the use of NSAIDs could then be very efficient, in order to decrease inflammation whilst the synovial fluid is cleared of most debris.

This hypothesis is in agreement with the odd finding by Bove et al. that NSAIDs worked well on day 14. Indeed, they used a 1-mg injection of MIA (instead of the 2 mg used in my studies). This technique was shown to give a slower onset of the chronic pain state. It could then be argued that following their treatment injection on day 14, the chronic nociceptive phase had not been reached yet: the initial inflammation phase might have been less intense following the injection of 1 mg of MIA, but the cartilage deterioration would also take longer. As a result, cartilage debris could be released for a longer period of time, which would result in a more delayed inflammation period, whilst the chronic nociceptive joint pain develops, hence the higher sensitivity to NSAIDs on day 14.

#### **4.4.3 *Validation of the use of the incapacitance tester to assess weight-bearing changes***

The use of the incapacitance tester in the MIA model of OA pain has been validated, along with its usefulness in studying various drug effects. Indeed, it had been used previously to assess weight-bearing changes in the MIA model (S. E. Bove et al., 2003; R. Combe et al., 2004; J. Fernihough et al., 2004; J. D. Pomonis et al., 2005), but only became available to me towards the end of this study about the role of inflammation. It will be used more extensively in the last behavioural chapter comparing the effects of a novel peripheral opioid receptor agonist to morphine and a control (Chapter 7). I had to decide which protocol to adopt as the literature reports were very variable in measures: either one three-second reading (J. Fernihough et al., 2004), one five-second reading (S. P. Ivanavicius et al., 2007), three five-second readings (S. E. Bove et al., 2003) or three three-second readings (J. D. Pomonis et al., 2005) were taken before being averaged. I chose to take three three-second reading to ensure the reproducibility and reliability of the measurements, as I noticed that a five-second period was too long to get a consistent reading because of the animals' tendencies not to stay in place in their cage. Indeed, the animals sometimes moved whilst some readings were taken, resulting in abnormal values (in comparison to the other two readings for example). Without those three readings, there would be too much variability and it would be more difficult to interpret the effects of a drug. Finally, we need to bear in mind that rats are quadrupedal animals and are different

from humans. Standing up in the small Perspex box was somewhat unusual and stressful for them, so the habituation process was very important for this device.

In the logic of finding a better cure for OA, or at least the optimal treatment for OA patients, we need to discuss the advantages and side-effects of the different classes of drugs used in this study.

#### **4.4.4 Advantages and side-effects associated with each class of drugs**

##### **4.4.4.1 Glucocorticoids**

Glucocorticoids, through their inhibition of PLA2 and IL-6 formation, have a powerful anti-inflammatory effect and are not very toxic when given acutely, on a short-term basis. This is why they are often used in emergency situations such as the treatment of anaphylactic shock (e.g. hydrocortisone). They are also used as “infiltrations” and can have a rapid but short-lived effect on pain. It can be particularly efficient to overcome a painful episode.

More interestingly, when given therapeutically to arthritic rats, they have been found to diminish joint destruction histologically (E. Larsson et al., 2004).

However, mainly because corticosteroid drugs mimic the effects of endogenous hormones whose production is physiological, they cannot be used constantly. It is indeed important to distinguish between their physiological effects (they can be used in replacement therapy) and their pharmacological effects.

One of the well known iatrogenic disease inducible by long-term glucocorticoid use is Cushing's syndrome, also called hypercortisolism or hyperadrenocorticism. It is an endocrine disorder caused by high levels of cortisol in the blood and its aetiology is very diverse as it can also be caused by any factor dysregulating the hypothalamic-pituitary-adrenal axis such as tumours, including primary pituitary adenoma (known as Cushing's disease), primary adrenal hyperplasia or neoplasia, and ectopic ACTH production due to a cancer cell. Cortisol is physiologically released from the adrenal glands in response to ACTH itself released from the pituitary gland. In Cushing's syndrome, ACTH levels will drop due to the negative feedback induced by the high levels of glucocorticoids on the hypothalamic-pituitary-adrenal axis.

Cushing's syndrome, which is also a relatively common condition in domestic dogs and horses, is characterised for example by abnormal fat deposition. Symptoms indeed include rapid weight gain, particularly of the trunk and face with sparing of the limbs, a round face often referred to as a "moon face", excess sweating, telangiectasia (dilation of capillaries), thinning of the skin (which causes easy bruising) and other mucous membranes, purple or red striae (also caused by thinning of the skin) on the trunk, buttocks, arms, legs or breasts, proximal muscle weakness (hips, shoulders), and hirsutism (facial male-pattern hair growth). However, it can have much more serious side-effects as excessive levels of glucocorticoids could also affect other hormonal systems and cause insomnia, reduced libido, impotence, amenorrhoea and infertility among others. Other psychological disturbances are also reported, ranging from euphoria to psychosis, in association with depression and anxiety. Other symptoms could also be caused by disturbance of sodium/water retention system through the mineralocorticoid effect of some glucocorticoid, causing persistent hypertension. This list is not exhaustive and shows the reason why glucocorticoids cannot be used for long periods of time.

In our specific case, the intraarticular injections of methylprednisolone in the knee are not recommended more than two or three times a year maximum. It is not really because of the systemic side-effects, but more because of the potential risks of infection associated with any injection. Indeed, the knee capsule, because of its specific closed structure, would be the worst place to have an infection: septic arthritis is well known to be very difficult to treat and it is often accompanied by serious joint deterioration. In the presence of the corticosteroid, the immune response would be lowered and it would be difficult to see the first signs of infection, which start with inflammation, because of the action of the drug and the side-effects associated with the injection itself. The pathogens could then proliferate and trigger irremediable damage by the time an adequate treatment be provided.

#### 4.4.4.2 TNF-alpha antagonists

As was detailed earlier, TNF-alpha antagonists act on the immune response by causing suppression. They are often used in inflammatory auto-immune diseases, such as RA in the case of etanercept. The big difference with glucocorticoids is that the treatment on those TNF-alpha inhibitors is a chronic one, until the symptoms improve. A 25 mg dose of Enbrel™, injected s.c. twice weekly, is indicated in adults for example.

The great advantage of such a treatment is that it can be stopped at any time and the different tools developed are very specific to TNF-alpha and nothing else. This explains the high cost of this new technology. However this is only a minor disadvantage to using it.

Indeed when TNF-alpha's actions are blocked, the responses of the immune system to infections will be altered. Consequently, etanercept treatment may worsen or increase the likelihood of infections, and patients with serious infections should not receive such treatment; treatment should also be discontinued if a patient develops a serious infection, and it should be monitored closely in patients prone to infection, such as those with advanced or poorly controlled diabetes.

Another direct implication of etanercept's mode of action is that any vaccination, in particular in children, should be done before starting the treatment.

Since etanercept is a relatively new drug, there is limited information on long-term risks associated with its use. Some reported associated conditions may or may not be related to etanercept. For example, there have been reports of multiple sclerosis, myelitis, optic neuritis in patients using the drug. Thus etanercept is not recommended for persons with pre-existing disease of the central nervous system (brain and/or spinal cord) or for those with multiple sclerosis, myelitis, or optic neuritis (Enbrel™ leaflet, Wyeth).

Because of all these side-effects, we have to remember that a treatment for any disease is the result of the acceptance of a benefit/risk ratio. For these reasons, it is unlikely that etanercept be used in OA or in inflammatory diseases other than systemic auto-immune disabling ones. It was solely used to assess the role of TNF-alpha at a certain point in the induction of our OA model.

#### 4.4.4.3 NSAIDs: the ideal COX inhibitor

The main objective of treatment in OA patients is symptomatic, aiming at decreasing the intensity and/or frequency of painful episodes. This can be achieved through the use of NSAIDs. Meloxicam has a selectivity ratio in favour of a bigger inhibition of COX-2, which limits the side-effects due to the inhibition of the physiological COX-1.

#### **A protective action on joint and cartilage?**

Apart from the advantage of that selectivity, and that meloxicam is available in a very palatable form, which can prove very useful in animals, there is some evidence that meloxicam, unlike other NSAIDs such as indomethacin, does not inhibit the synthesis of proteoglycans at very high concentrations both *in vitro* and *in vivo* in dogs (K. D. Rainsford et al., 1999).

Indeed the idea of certain NSAIDs having a promoting or accelerating effect on OA has been shown extensively with indomethacin in humans (S. Rashad et al., 1989), which raised the same concerns in animals, in particular dogs. Some studies later showed that long-term administration of NSAIDs such as salicylates and indomethacin in a model of OA induced by cruciate ligament transection in dogs accelerated cartilage destruction, associated with some reduced proteoglycans synthesis and content in cartilage (K. D. Brandt and M. J. Palmoski, 1984). However, other NSAIDs do not have these negative effects on cartilage, but could even have protective effects against cartilage degeneration in OA because of their inhibitory effects on MMPs that are involved in that destructive process (K. D. Brandt and M. J. Palmoski, 1984). The NSAID carprofen could for example have a protective effect on the structural changes and on the abnormal subchondral bone metabolism induced by experimental OA in dogs (J. P. Pelletier et al., 1999).

It would thus be very sensible to adapt the prescription of NSAIDs based on these considerations when they are available.

#### **No ulcerogenic effect on gastrointestinal tract**

The use of NSAIDs having a preferential COX-2 activity could also be a considerable advantage, given that COX inhibitors are a major iatrogenic cause of death in patients by causing gastrointestinal ulcers leading to bleeding for example.

Using a COX inhibitor drug like meloxicam having a preferential effect on COX-2 can help to circumvent this important side-effect. However, some dosing problems still remain. For example, the selectivity ratio COX-1/COX-2 is dose-dependent, and by increasing the dose, it is very likely that the effect on COX-1 will increase as well.

Meloxicam in humans was shown to have an efficacy dose range between 7.5 and 22.5 mg daily. It was well tolerated with fewer gastrointestinal side-effects at the daily dose of 7.5 mg compared to other NSAIDs, although higher doses have not been studied (C. J. Hawkey, 1999).

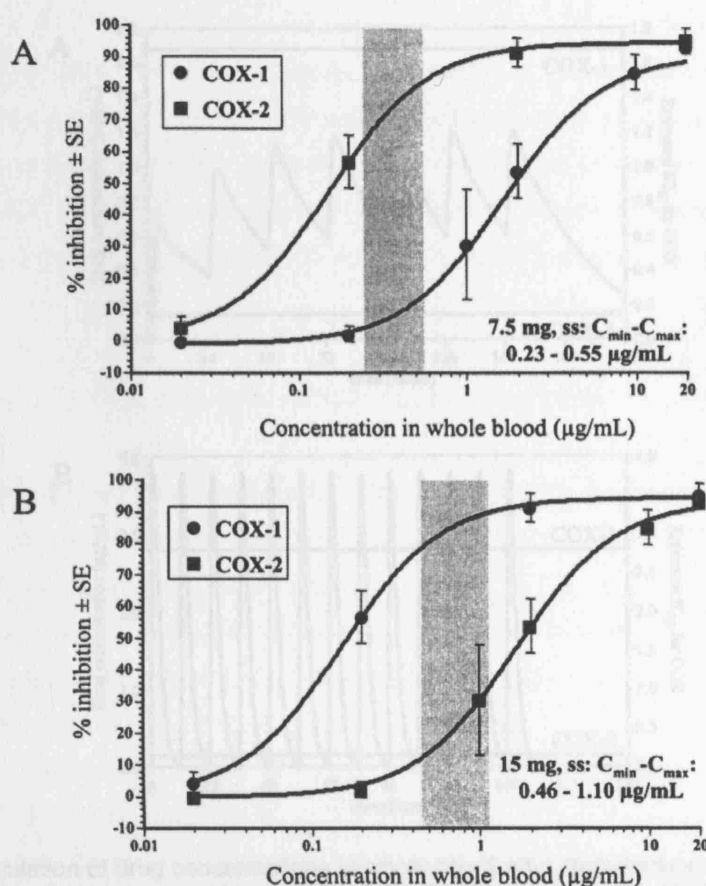


Figure 4.12. Comparison of concentration-response curves for COX-1 and COX-2 inhibition by meloxicam with therapeutic concentrations at the human recommended doses of (A) 7.5 mg/day and (B) 15 mg/day. Drug concentrations in whole blood are calculated from plasma concentrations, assuming drug concentrations in red cells are negligible and that hematocrit is 45%. From (M. Pairet and J. van Ryn, 1998).

When looking at Figure 4.12, it can be seen that from those simulations, the dose of 7.5 mg/day of meloxicam is sparing COX-1. However, 15 mg/day not only gives an inhibition of COX-2 over 90%, but it also causes an expected inhibition of COX-1 in the order of 30%. These simulations are important for compounds with relative selectivity for COX-2 such as etodolac, nimesulide and meloxicam, showing the loss of their COX-1 sparing effect at higher doses.

We can also compare simulations of administering meloxicam 15 mg once daily and nimesulide 200 mg twice daily. Figure 4.13 showing the importance of the dosing regimen used.

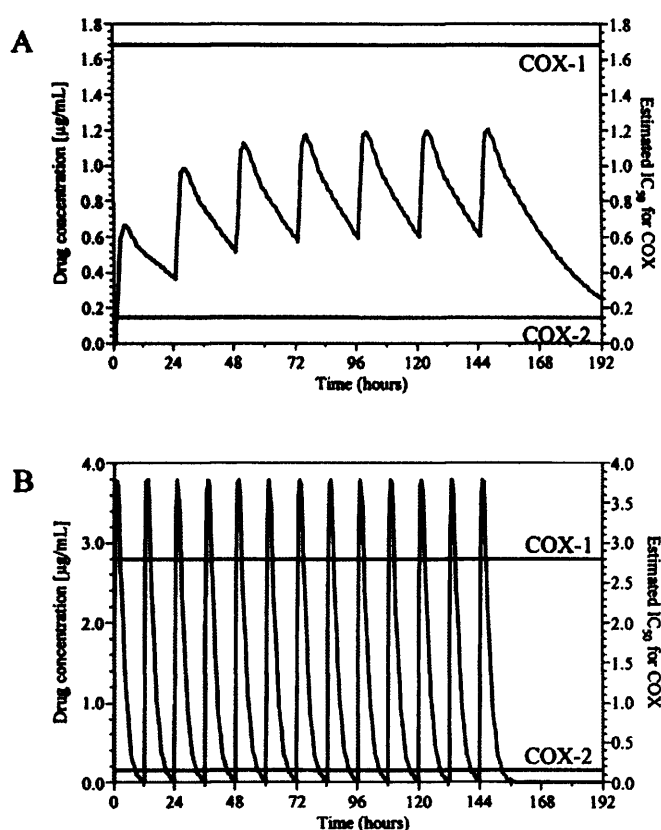


Figure 4.13. Simulation of drug concentrations in whole blood after repeatedly giving meloxicam 15 mg once daily (A) and nimesulide 200 mg twice daily (B). Comparison with  $IC_{50}$  values for COX-1 and COX-2. From (M. Pairet and J. van Ryn, 1998).

Indeed, ideally a flat plasma concentration curve is necessary to maintain drug concentrations above the effective inhibitory concentrations for COX-2 but below the effective inhibitory concentrations for COX-1, which would be the case for meloxicam but not for nimesulide at the given doses.

However, these are *in vitro* experiments and they may not reflect what is happening *in vivo*, in the synovial fluid for example.

### Similar pharmacological profile between species

Another interesting feature of meloxicam was its similar pharmacokinetic profile in both rats and humans, which could be very useful in making clinical predictions. However, this was very unusual, as the profiles between humans and other species (dog, mice, etc.) were different (U. Busch et al., 1998). This is also variable with the NSAID studied.



**Low cost**

The ideal COX inhibitor would be cheap. It is important in human medicine (cost/benefit ratio), but it plays an even major role in veterinary medicine. Meloxicam, for example, is very expensive to administer to a large dog with hip OA in a chronic manner, which can sometimes be full of consequences as some owners might not be able to afford such a treatment in the long term. Treating a 500-kg race horse following an injury would even be more costly.

**4.4.5 COX-2 specific inhibitors, the panacea for OA pain?**

The final issue I wanted to discuss is the use of the newly developed COX-2 specific inhibitors. Indeed, we all heard about the sad fate of rofecoxib (or the blockbuster-to-be arthritis drug Vioxx<sup>TM</sup>), when in September 2004, Merck & Co., announced the voluntary, worldwide recall of Vioxx<sup>TM</sup> after a clinical trial confirmed previous studies linking Vioxx<sup>TM</sup> to serious cardiovascular problems, including heart attack and stroke. This withdrawal marked one of the biggest drug recall in history.

Are COX-2 specific inhibitors the panacea, the cure to inflammatory conditions and arthritis?

COX-2 specific inhibitors mainly have the advantage of not interacting with the constitutive constitutively expressed COX-1 in the gastrointestinal tract for example. Although they could be very useful in theory, they are very expensive drugs. In addition, some unexpected side-effects have seriously impacted on their share on the NSAIDs market.

Indeed, all of these compounds can interfere with prostaglandin production in many organs such as the kidney, in relation with increased blood pressure and water retention caused by COX-2 inhibitors (J. I. Schwartz et al., 2002). Blood pressure control could also be affected by their activity in hypertensive patients (W. B. White et al., 2002).

The selective inhibition of COX-2 may also be a risk factor in patients at risk for cardiovascular thromboembolic reactions. Indeed, endothelium-derived prostacyclin was shown to be partly produced by COX-2 (G. A. FitzGerald and C. Patrono, 2001), and the pro-aggregatory thromboxane by COX-1 in blood platelets. The disruption of this balance could result in an increased risk of thromboembolic reactions at high doses of selective COX-2 inhibitors in some patients known to be at increased cardiovascular danger, for example, those suffering from RA (C. Bombardier et al., 2000) and those undergoing coronary surgery (N. A. Nussmeier et al., 2005). Finally, it has recently been shown that

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COX-2-selective and non-selective inhibitors may increase the risk of myocardial infarction and stroke (R. S. Bresalier et al., 2005; S. D. Solomon et al., 2005; ADAPT, 2006).

## 4.5 Conclusion

I have proposed a mechanism for the role of inflammation in this model of OA. Whilst inflammation seemed to play a major role during the first week post OA-induction, its role looked limited during the second week. This corresponds to the transition between the inflammation caused by the MIA injection and the start of the chronic nociceptive pain state from day 14, which was in agreement with previous histological studies. From day 14, inflammation was unlikely to be present constantly, as in humans where it is believed that some osteocartilaginous fragments could be released from time to time, initiating the inflammatory process, which would then be maintained until various healing mechanisms are finished. This could explain why NSAID treatment gave variable results. In this context, it can be postulated that NSAID would be very useful during a painful inflammatory episode, but would not work on chronic ongoing nociceptive OA pain *sensu stricto*.

I have previously shown the efficacy of morphine and gabapentin on the MIA-induced pain state: I am now going to investigate the role of neuropathy to see to what extent it is important in this model of OA pain.

**Chapter 5. An immunohistochemical study investigating the role of  
neuropathy in the MIA model of OA pain**

## 5.1 Introduction

Following the positive studies with morphine and gabapentin, and based on the results relating to the presence of inflammation, a natural progression was to study the contribution of a potential neuropathic component of pain in this model. Contrary to inflammation, there was no pharmacological agent available specifically acting on processes related to neuropathy. Indeed, one of the few drugs licensed for the treatment of neuropathic pain, gabapentin, was originally designed for the treatment of epilepsy, yet it had some effects on behavioural measures in this osteoarthritis pain model as described in Chapter 3.

I thus decided to use a molecular approach to investigate the changes in the expression of molecular markers specific for nerve damage, in primary afferents – in particular in their dorsal root ganglia, DRG - following the induction of OA.

### 5.1.1 Markers studied

There is a great diversity of nociceptors present on primary afferents, and schemes whereby their fibres can be classified based on some molecular markers such as NF200 in large myelinated fibres, CGRP in peptidergic fibres or IB-4 in non-peptidergic fibres.

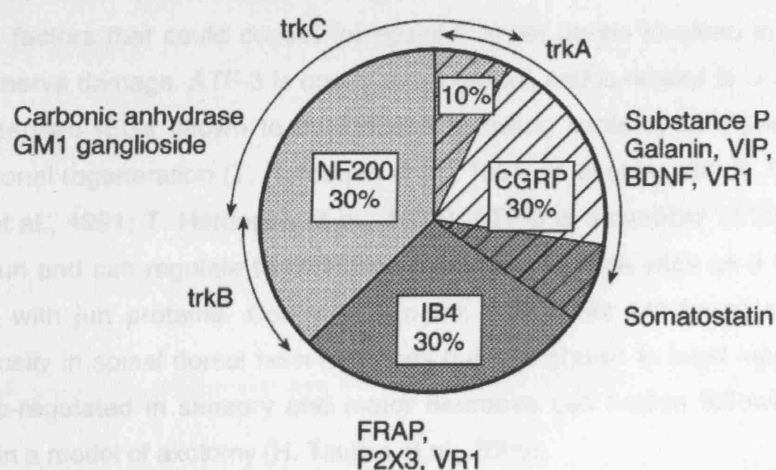


Figure 5.1. The different neuronal cell populations residing in the dorsal root ganglia. The percentages represent the size of the population in relation to the total neuronal population in the dorsal root ganglion. Large diameter cells give rise to myelinated axons (presumably A-beta fibres) and have high levels of neurofilament (NF200). Small cells, with mainly unmyelinated axons, comprise the remaining two populations. One population of small cells constitutively synthesises

neuropeptides such as CGRP and responds to the growth factor NGF, whereas the other expresses the lectin IB4 (isolectin B 4) and responds to GDNF. Both small cell populations express the VR-1 receptor and are thought to be nociceptors. It should be noted that there is overlap between populations. BDNF, brain-derived neurotrophic factor; FRAP, fluoride-resistant acid phosphatase; TrkA, tyrosine kinase receptor A; TrkC, tyrosine kinase receptor C; TRPV1, vanilloid receptor 1; VIP, vasoactive intestinal polypeptide (From B. L. Kidd and L. A. Urban, 2001, Provided by J. V. Priestley; J. V. Priestley et al., 2002).

In the MIA model of OA, it was shown that the expression of CGRP (calcitonin gene related peptide) and TRPV1, the vanilloid receptor 1 or capsaicin receptor, was increased in DRGs from MIA-treated rats compared to sham rats (J. Fernihough et al., 2005). ATF-3 (activating transcription factor 3), a marker of neuronal damage, was also studied and I am going to detail the characteristics of different markers I used.

#### 5.1.1.1 ATF-3, a unique neuronal marker of nerve injury in the nervous system

Activating transcription factor 3 (ATF-3), a member of ATF/CREB family of transcription factors sharing many characteristics of IEGs, was shown to be induced in a variety of stressed tissue. Indeed, following peripheral nerve injury, various regeneration and restoration mechanisms of lesioned nerves can occur, involving changes in gene expression in the affected neurones as well as in glial cells. IEGs could well play a crucial role in the nervous system plasticity and long-term changes incurred, for example in central sensitisation (cf. Chapter 1. Introduction). Those IEGs could encode for transcription factors that could control for specific target genes involved in the neuronal response to nerve damage. ATF-3 is one of those genes, and is related to c-jun, one of the best characterised IEGs shown to be induced following axotomy and presumed to be related to axonal regeneration (T. Herdegen et al., 1991; R. Jenkins and S. P. Hunt, 1991; J. D. Leah et al., 1991; T. Herdegen et al., 1992). ATF-3 is a member of the same super family as c-jun and can regulate transcription by binding to DNA sites as a homodimer or heterodimer with jun proteins. Contrary to c-jun, ATF3 was not found to be induced transsynaptically in spinal dorsal horn neurones but was shown to label injured neurones as it was up-regulated in sensory and motor neurones cell bodies following peripheral nerve injury in a model of axotomy (H. Tsujino et al., 2000).

The expression of ATF-3 was also found to be increased in a model of cancer-induced bone pain (C. M. Peters et al., 2005; M. A. Sevcik et al., 2005).

More recently, the expression of ATF-3 (activating transcription factor 3), a marker of neuronal damage, was studied and used to suggest an early phase neuropathy in the L5 innervation territory of the knee following OA induction with MIA (S. P. Ivanavicius et al., 2007).

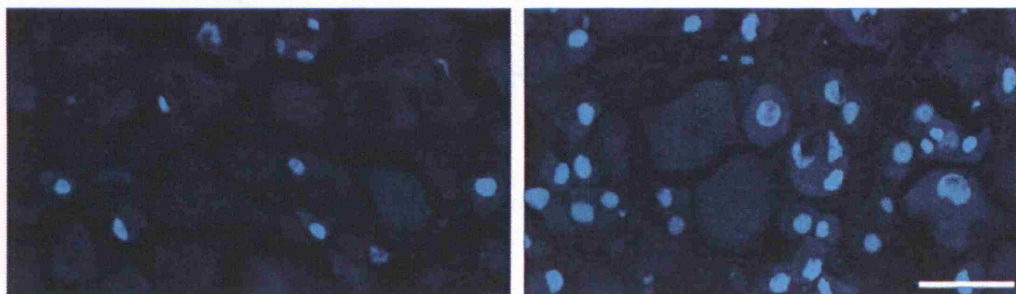


Figure 5.2. Comparison of ATF-3 immunoreactive sensory neurones within the mouse DRG following tumor implantation and femoral nerve transection. Fourteen days following the injection and confinement of 2472 sarcoma cells into the intramedullary space of the mouse femur approximately 17% of L2 DRG neurons expressed ATF3 (left) compared to femoral nerve transection (right) where approximately 44% of the total L2 DRG neurons expressed the axonal injury marker ATF3. Note the higher intensity and number of ATF3-IR sensory neurons observed in ipsilateral L2 DRG from femoral nerve transected animals. Scale bar = 40  $\mu$ m (C. M. Peters et al., 2005).

#### 5.1.1.2 Beta-III tubulin, a structural neuronal marker

In order to be able to quantify the expression of ATF-3, the structural marker beta-III tubulin (Clone Tuj1), which identifies microtubules, was also used. This antibody was raised against microtubules derived from rat brain and recognises a specific peptide epitope. It was well characterised and was shown to be highly reactive to neurone specific Class III beta-tubulin (beta III). More importantly, Tuj1 does not identify beta-tubulin found in glial cells.

#### 5.1.2 *Innervation of the knee*

Although it is generally well accepted that the hind limb is innervated by the femoral, obturator and sciatic nerves that all have lower branches, the composition of the lumbosacral plexus and the spinal nerve distribution can vary a lot between species. For example, in humans the sciatic nerve would mainly originate from spinal nerves L5, S1 and S2, whereas in Sprague-Dawley rats, it would be more L4, L5 with sometimes a contribution from L6 (F. Asato et al., 2000), the latter being absent in humans.

Dermatomes, the charts indicating the distribution of segmental cutaneous sensory nerves in the skin of vertebrates, have also already been studied in rats (Y. Takahashi and Y. Nakajima, 1996), suggesting an innervation by L3 to L5 (Figure below).

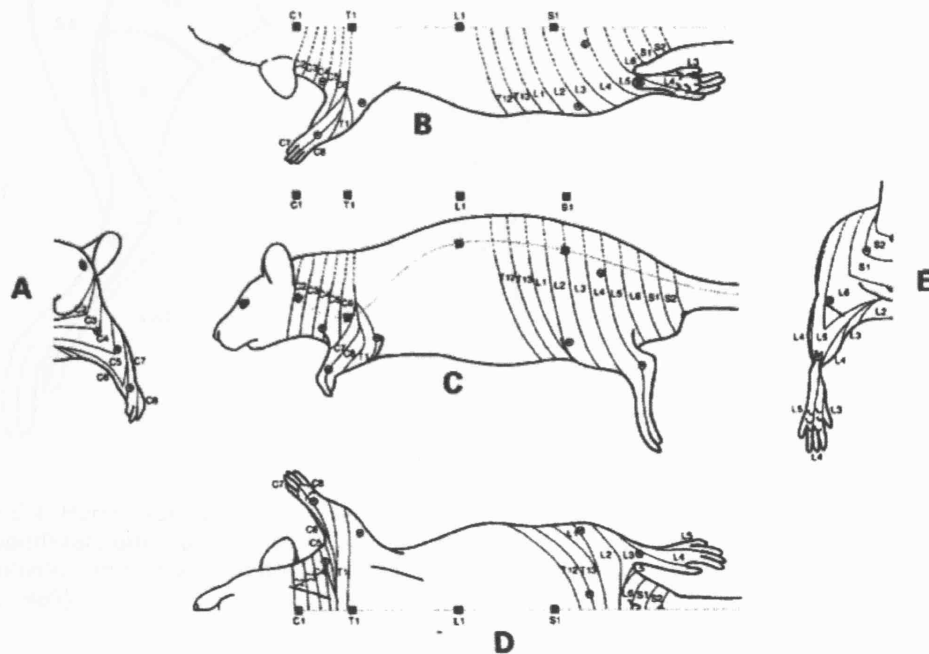


Figure 5.3. Composite dermatome charts. A: anterior; B: dorsal; C: lateral; D: ventral; E: posterior aspects. Open circles indicate the position of shoulder, elbow, wrist, hip, knee and ankle joints. (Y. Takahashi and Y. Nakajima, 1996)

In addition, the somatotopic organisation of primary afferent terminals and the peripheral cutaneous receptive fields in the rats' hind limb have also been investigated (J. E. Swett and C. J. Woolf, 1985) showing that the saphenous nerve branching from the femoral nerve, and the sural and lateral sural branching from the sciatic nerve were mainly responsible for the innervation of the skin in the knee region (Figure 5.4).

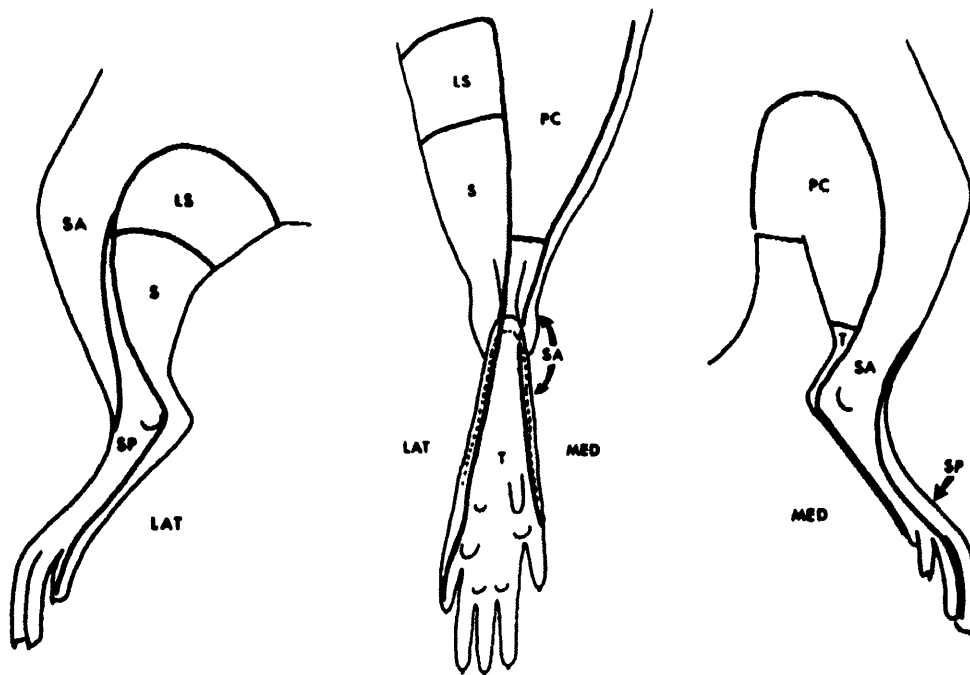


Figure 5.4. Peripheral cutaneous receptive fields on the rat's hind limb. S: sural, LS: lateral sural; SP: superficial peroneal; T: tibial; (sciatic nerve=S+LS+SP+T); PC: posterior cutaneous of the thigh or cutaneous femoralis posterior; SA: saphenous, branch of femoral nerve (J. E. Swett and C. J. Woolf, 1985).

However, these studies were not very informative about the knee innervation. I could have followed Hilton's law according to which "The nerve supplying a joint supplies also the muscles which move the joint and the skin covering the articular insertion of those muscles", but this law does not allow for a dichotomy between sensory and motor neurones. It could have resulted in the study of all DRGs from around L1-L2 to L6, without knowing what proportion of the changes in the expression of the marker were caused by the knee damage itself.

Using retrograde tracing of knee joint afferent nerves with Fluoro-Gold in female Wistar rats, Salo et al. (1997) showed that the afferents were found distributed from L1-L5 ganglia, with the great majority between L3 and L4 ganglia (Figure 5.5).



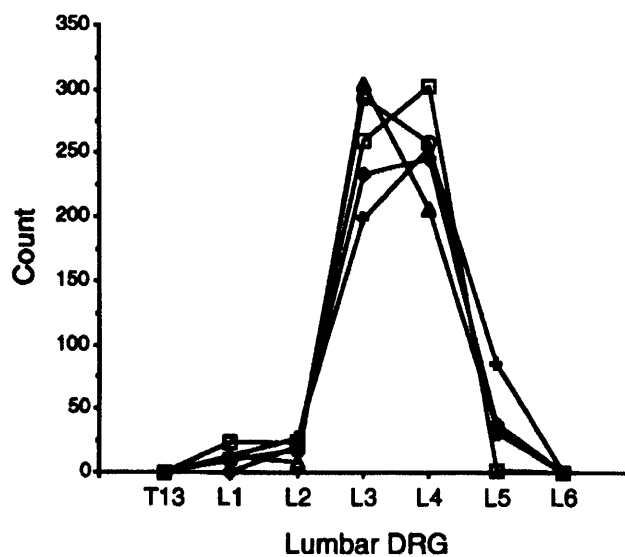


Figure 5.5. Distribution of Fluoro-Gold labelled knee joint afferents in the lumbar dorsal root ganglia (DRGs). Each set of joined points represents the results of 1 knee joint injection in 1 animal. (From P. T. Salo and E. Theriault, 1997)

In our same model, Fernighough et al. (2005) looked at the expression of CGRP and TRPV1 in the L4 DRG only, while Ivanavicius et al. (2007) looked at the expression of ATF-3 in the L4 and L5 DRGs.

Based on this evidence, it appeared sensible to look at the changes at least in the L3 and L4 DRGs. However, in order to confirm the correct segments and to measure changes in direct relation with the knee, I decided to utilise a retrograde labelling (or back-labelling) technique using a fluorescent marker.

### 5.1.3 *Immunofluorescent retrograde labelling*

The idea here was to inject a fluorescent neuronal marker before or at the same time as OA induction directly into the knee capsule to ensure that the DRGs that we would be studying would have received some input from the knee itself. Indeed, the fluorescent marker would then be taken up by the primary afferent fibres innervating the knee joint, and transported back to the DRGs where the cell bodies would be stained and visible directly using a microscope under UV light. As a result, some triple labelling studies, involving the fluorescent marker, beta III tubulin and ATF-3 for example could be performed and it would be possible to study the proportion of ATF-3 positive cells from the

cells that actually innervated the knee joint, i.e. the neurones that would have incorporated the fluorescent dye.

Fluoro-Gold (FG) had been used previously. However, when doing a pilot study and looking at the effects of the intraarticular injection of a fluorescent marker, the marking resulting from Fast Blue (FB), a commonly used neuronal tracer, looked much clearer, brighter and was more intense, with cells being better delimited than with FG. Quantifying FB positive cells was thus easier than FG ones and I decided to use Fast Blue.

Fast Blue fluorescent dye is commonly used as a neuronal tracer and is sometimes referred to as a hydrophilic retrograde tracer. It is soluble in water and lower alcohols.

The excitation wavelength is 365 nm and the emission wavelength 420 nm.

FB has been studied with retrograde labelling of motoneurones and was shown to be a more persistent dye, as well as a non toxic one, which made it a marker of choice over Fluoro-Gold, Mini-Ruby, Fluoro-Ruby and Fluoro-Emerald in long-term motoneurones labelling (L. Novikova et al., 1997).

#### 5.1.4 *Alpha-2-delta receptor VGCC subunits*

The effects of gabapentin in this model of OA were previously discussed. Gabapentin has been previously shown to bind to the alpha-2-delta type-1 subunit of neuronal voltage-gated calcium channels (VGCC) (N. S. Gee et al., 1996; M. J. Field et al., 2000; C. P. Taylor, 2004; F. Bian et al., 2006). It is used in the treatment of both clinical and experimental painful peripheral neuropathies (Reviewed in P. J. Wiffen et al., 2005).

The alpha-2-delta subunit, a glycoprotein consisting of covalently linked alpha-2- and delta- peptides encoded by the same gene (K. S. De Jongh et al., 1990), is important for the functional assembly of the voltage-gated calcium channels. Most of the alpha-2-delta subunit is extracellular, with only one transmembrane domain and five intracellular C-terminal amino acids. The extracellular domain of the subunit was shown to be important *in vitro* for channel function and the coexpression of the alpha-2-delta subunit with other calcium channel subunits resulting in enhanced calcium channel currents. This is accompanied by an increase in both the number of binding sites and their affinity for omega-conotoxin, a ligand for neuronal voltage-gated calcium channels (Y. Mori et al., 1991; M. E. Williams et al., 1992; P. F. Brust et al., 1993; C. A. Gurnett et al., 1996). Three

genes encoding the alpha-2-delta-1, alpha-2-delta-2, and alpha-2-delta-3 subunits, respectively, have been identified in mice (N. Klugbauer et al., 1999).

Levels of alpha-2-delta type-1 in the dorsal root ganglia (DRG) and dorsal spinal cord are increased in rats in models of post-traumatic and diabetic painful peripheral neuropathies (reviewed in (Z. D. Luo et al., 2002)). For that reason, I also examined the expression of the alpha-2-delta-1 subunit in the DRGs (L4 and L5+L6) and spinal cord of MIA-treated rats. DRGs from SNL rats were also used for comparison purposes.

## **5.2 Methods**

### **5.2.1 *Retrograde labelling and induction of OA***

In order to label in the DRGs the primary afferents coming from the knee, I used the fluorescent neuronal marker Fast Blue (FB) that would be taken up at the nerve terminals in the knee and transported up to the cell bodies.

The intraarticular injections were performed as previously described. Briefly, after clipping and disinfection of the knee, injections into the knee capsule using a 27 G needle under 2% halothane in oxygen anaesthesia were made.

Then I performed two different studies, study A and study B, using different protocols.

In study A, 10 µl of 1% FB was administered intraarticularly as previously described, 6 days prior to the MIA injection (day -6). 6 days later, on day 0, the animals then received the usual MIA intraarticular injection (2 mg of MIA in 25 µl of saline) to induce OA pain.

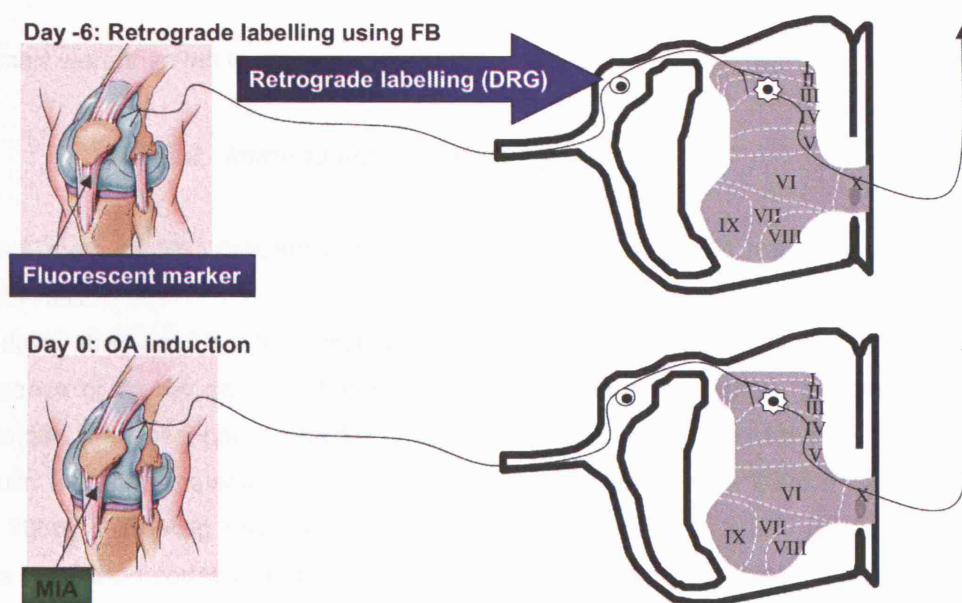


Figure 5.6. Retrograde labelling of DRGs using FB 6 days before OA induction with MIA (study A).

In study B, in order to try to refine the technique, 10  $\mu$ l of 1% FB were mixed with 15  $\mu$ l of saline containing 2 mg of MIA (reaching a final injection volume of 25  $\mu$ l) and administered them together in a single intraarticular injection on day 0.

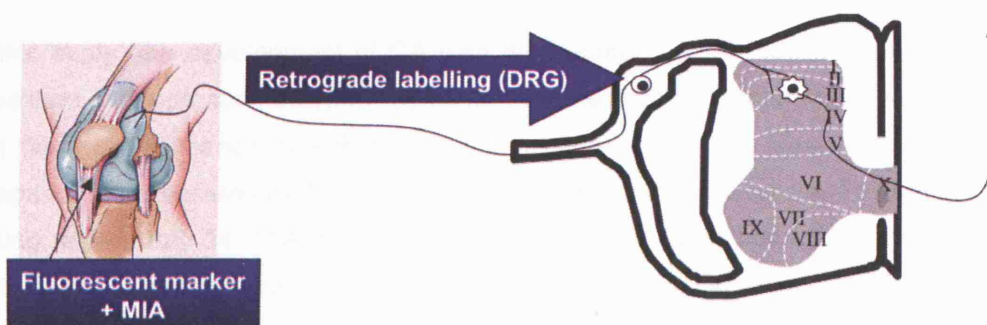


Figure 5.7. Retrograde labelling of DRGs innervating the knee following a single injection of FB and MIA into the joint on day 0 (study B).

In order to comply with the “three Rs” rule of animal testing (reduction, replacement, refinement) by limiting the number of animals used, contralateral knee joints were used as controls for these studies: they were injected on the same day with the same volume of saline with FB only, i.e. either 10  $\mu$ l (1 % FB) or 25  $\mu$ l (10  $\mu$ l 1 % FB + 15  $\mu$ l saline) for study A and study B, respectively.

Animals were then left to recover at room temperature.

### **5.2.2 *Immunohistochemistry***

These methods are detailed in the main methods section and they are only presented briefly here.

14 days after the FB+MIA injection, following the last behavioural testing verifying the presence of an OA pain-like behaviour, animals were transcardially perfused and DRGs collected then sectioned. The DRG sections were then stained for ATF-3 and beta III tubulin following previously described protocols (S. Pezet et al., 2006; P. J. Shortland et al., 2006). Following microscope photography of slides, ATF-3 and FB immunoreactive cells were then counted and expressed as a percentage of the total of neurones (marked by beta-III tubulin).

An ATF-3 positive control staining was also done on L5 DRG sections from an animal having undergone a spinal nerve ligation (SNL) 7 days prior to the staining (kindly provided by Dr Sophie Pezet, King's College London), in order to verify the validity of the technique used.

### **5.2.3 *Measurement of mRNA levels by real-time qPCR***

In this study, the development of OA pain development was assessed using previously described methods such as von Frey hairs, acetone drop test, rotarod, as well as for the first time, the incapacitance tester. The use of the latter test was validated here by comparing pain behaviours from 4 MIA injected rats and 4 sham rats. Following the last testing day on day 14, MIA-treated animals were decapitated and L4, L5 and L6 DRGs (both IL and CL) were harvested by Dr Wahida Rahman (UCL) and kept at  $-80^{\circ}\text{C}$ .

The DRGs coming from the four animals were grouped and pooled as follows:

- L4 IL
- L4 CL
- L5+L6 IL
- L5+L6 CL.

A real-time quantitative polymerase chain reaction (qPCR) study was then performed by Dr Laurent Ferron and Claudia Bauer (UCL). It is a modified version of the classic PCR, with various cycles of amplification where the amount of DNA is measured after each cycle

of PCR by use of fluorescent markers in order to have a quantitative approach to DNA (or mRNA) levels. There is often confusion with RT-PCR (reverse transcription-polymerase chain reaction), the transcription of mRNA to DNA by a reverse transcriptase, which can be necessary when mRNA levels need to be studied using real-time qPCR.

Real time qPCR was used as it remains the most sensitive technique for the reliable detection of mRNA targets, and its application has become the most popular method of quantifying steady-state mRNA levels (S. A. Bustin, 2000). The technique measures fluorescent signals that are proportional to the amount of DNA produced during each PCR cycle (the more target there is, the greater the rise in fluorescence above background fluorescence). It comprises three steps, namely the reverse-transcriptase-dependent conversion of mRNA into the more stable cDNA, amplification of this cDNA using PCR, and finally detection and quantification of the PCR products using fluorescent reporter dyes. The use of probes labelled with different reporter dyes allows the detection and quantification of multiple target genes (i.e. genes for 5HT3A and 5HT3B receptors, as well as  $\alpha 2\delta 1$  and  $\alpha 2\delta 2$  subunits) in a single reaction.

Briefly, RNA was isolated using RNeasy columns (Qiagen), including an on-column DNase step. Reverse transcription was carried out on 1  $\mu$ g RNA using the iScript kit with random primers (BioRad, Hercules CA, USA). qPCR was performed with an iCycler (BioRad, Hercules CA, USA) using the iQ SYBR supermix. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA.

For each set of primers and for every experiment a standard curve was generated using a serial dilution of reverse-transcribed RNA combined from several samples. Data were normalised for expression of GAPDH mRNA.

The following qPCR primers were used:

- rat glyceraldehyde-3-phosphate dehydrogenase GAPDH (AF106860) 5'-ATGACTCTACCCACGGCAAG-3', 5'-CATACTCTGCACCAGCATCTC-3' ;
- rat  $\alpha 2\delta 1$  (NM012919) 5'-AGCCTATGTGCCATCAATTAC-3', 5'-AGTCATCCTCTTCCATTTC AAC-3' ;
- rat  $\alpha 2\delta 2$  (NM175592) 5'-CAGTGGTGGGTGTCAAAC-3', 5'-TACCTCGCAGTCCATCTC-3' .

This study was part of a larger study investigating the mRNA levels of those targets in other chronic pain models, including the spinal nerve ligation (SNL) model whose results will be shown for comparison purposes. The SNL model of neuropathic pain consists in

ligating the spinal nerves L5 and L6. The sciatic nerve is mainly composed of the spinal nerves L4 and L5, with L6 not entirely fused with this nerve in Sprague-Dawley rats (F. Asato et al., 2000).

## **5.3 Results**

### **5.3.1 *ATF-3, Study A***

Figure 5.8 shows the staining of DRG sections with ATF-3, FB and beta-III tubulin triple labelling in a MIA treated animal. We can clearly see how beta-III tubulin marked all neurones, whilst fewer neurones showed FB fluorescence, and only one neurone was immunoreactive to ATF-3. I chose this picture because of the triple-labelled neurone, however it was not representative of the general cell populations.

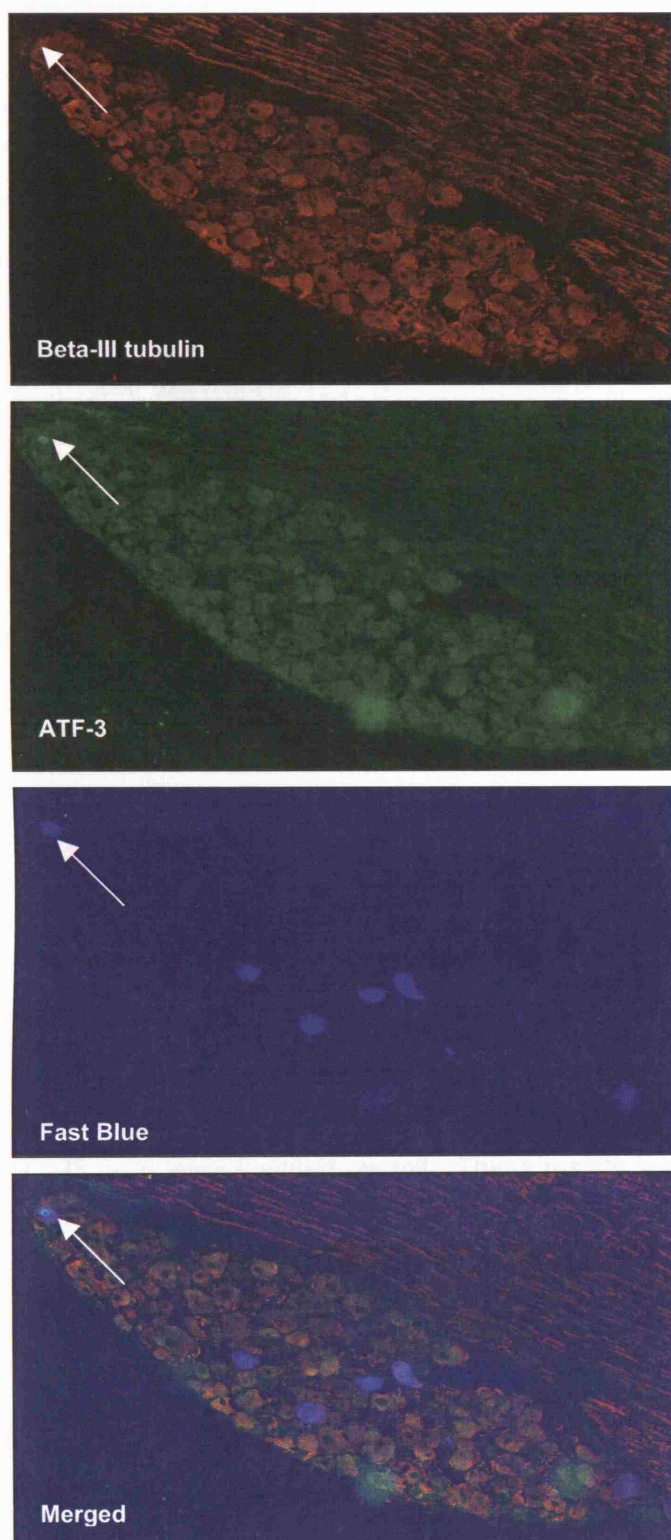


Figure 5.8. Example of L3 DRG section stained for beta-III tubulin (red), ATF-3 (green, positive staining shows as bright green nucleus), Fast Blue (blue) and the merged picture. The arrow points at a triple labelled neurone.



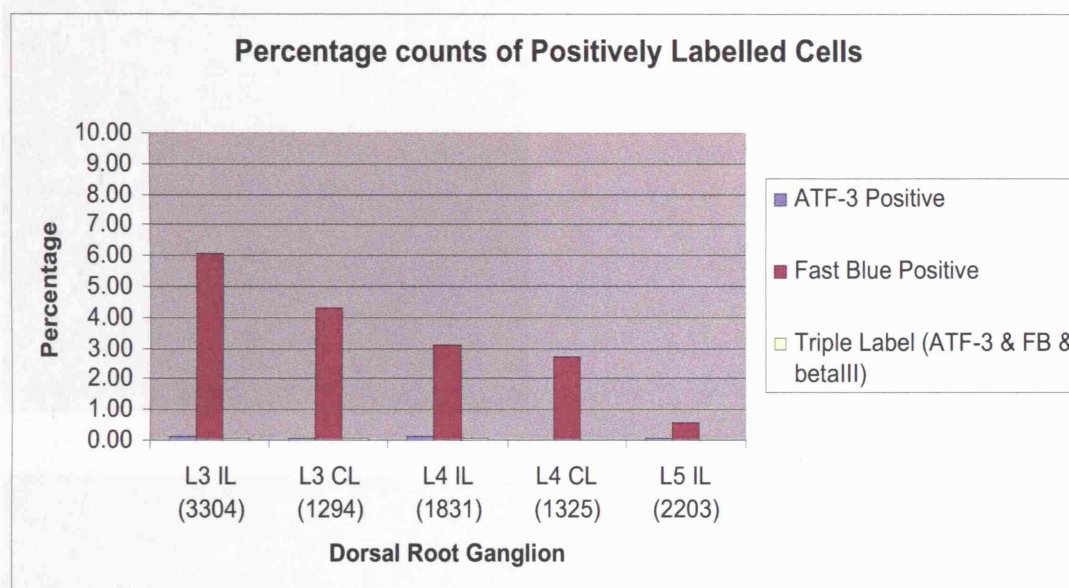


Figure 5.9. Percentage counts of ATF-3, Fast Blue and triple labelled cells at each lumbar dorsal root ganglion. The numbers in brackets denote the total number of neuronal cells counted for each spinal level using beta-III tubulin staining.

As expected, the largest percentages of FB cells were found in the L3 (6.05% IL, 4.33% CL) and at L4 (3.11% IL, 2.72% CL) DRGs, with only 0.55% of the cells in the IL L5 Fast Blue positive, which was in agreement with previous studies (P. T. Salo and E. Theriault, 1997).

The percentages of ATF-3 positive cells in DRG dissected from experimental animals were 0.12%, 0.08% and 0.09% for ipsilateral L3, L4 and L5 DRGs respectively, with only 3 triple labelled cells (i.e. positive for ATF-3, FB and beta-III tubulin) being found on IL L3-L5 (7,338 cells were counted overall). These results suggest therefore that there was little nerve damage in the animals in this study following MIA injection, and that even less nerve damage has occurred specifically in the knee joint (i.e. very few backlabelled cells were also ATF-3 positive).

One ATF-3 positive cell, which was also triple labelled, was found on the contralateral L3 slides, which could imply some nerve damage in the ipsilateral knee joint.

This very low level of ATF-3 can be compared to tissue from rats having undergone L5 SNL (spinal nerve ligation), with a level of 40.62% on the ipsilateral L5 DRG sections stained. The next figure shows a section from the SNL animal, with ATF-3 reactivity in the majority of cells counted (i.e. whose nucleus is visible after beta-III tubulin immunoreactivity), which was not the case for the MIA treated rats.

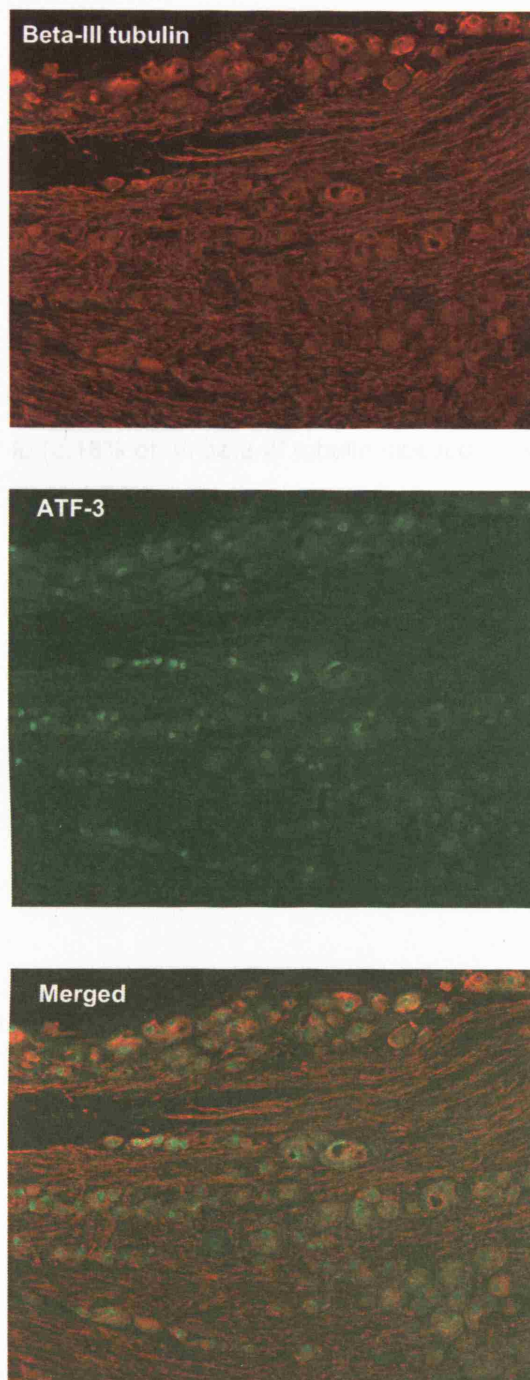


Figure 5.10. Example of section stained for ATF-3 coming from a positive control animal (Day 7 post-spinal nerve L5 ligation), DRG L5 ipsilateral to the L5 ligation. Tissue kindly provided by Sophie Pezet, King's College London.

### 5.3.2 *ATF-3, Study B*

In this study, FB and MIA were injected at the same time on day 0. An example of section after ATF-3 and beta-III tubulin staining is shown on Figure 5.11.

On this section of a L4 DRG ipsilateral to the injected side, there was no FB or ATF-3 immunoreactivity.

Those results were confirmed by the quantitative analysis performed (Figure 5.12), where I found no ATF-3 positive neurones in L3 IL, L3 CL, L4 IL, L4 CL and L5 CL, but only in L5 IL (0.18% of all beta-III tubulin labelled cells). The FB fluorescence was mainly found in L3 and L4 DRGs.

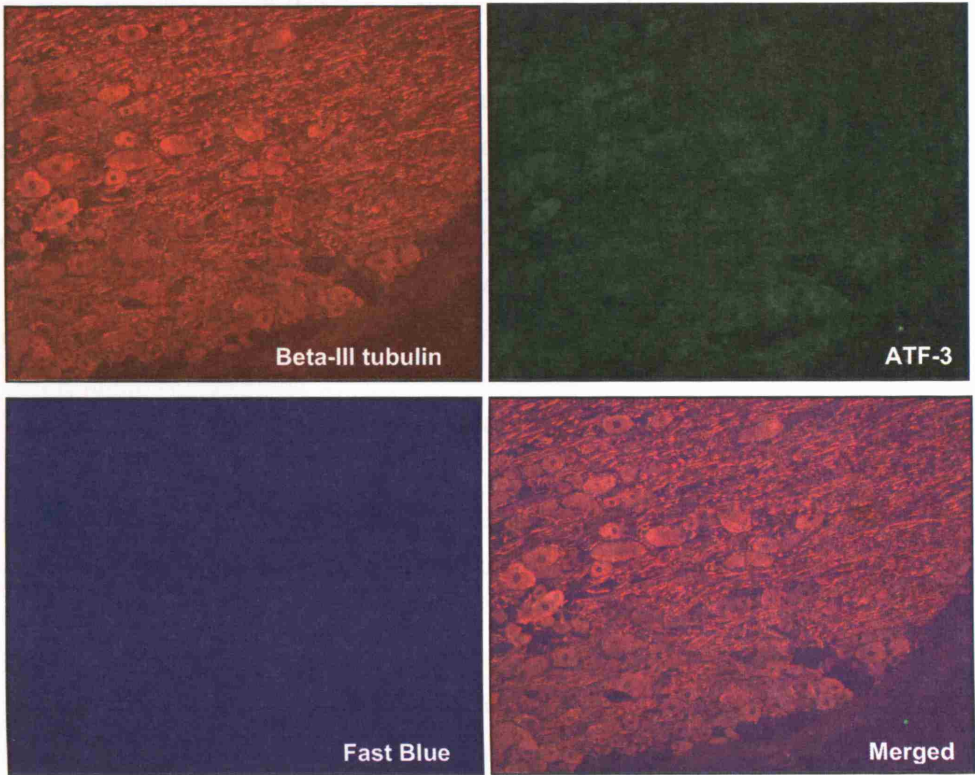


Figure 5.11. Typical section from a L4 DRG ipsilateral to the arthritic side, 14 days post MIA+FB injection animal Study B.

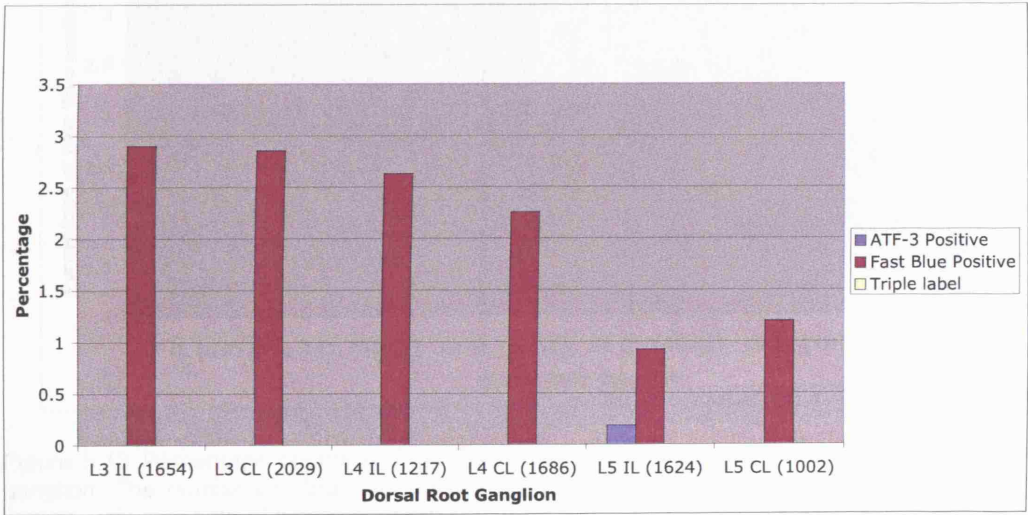


Figure 5.12. Percentage counts of ATF-3, Fast Blue and triple labelled cells at each lumbar dorsal root ganglion. The numbers in brackets denote the total number of neuronal cells counted for each spinal level using beta-III tubulin staining.



### 5.3.2.1 Conclusion of the ATF-3 studies

ATF-3 was expressed at very low levels (study A) and was even absent (study B) from sections of L3, L4 and L5 DRGs ipsilateral to the lesioned knee, suggesting the absence of nerve damage 14 days after MIA injection. Only the L5 DRG IL to the lesioned knee showed a low level of expression of ATF-3 in both studies (<0.2%).

The validity of the technique used was verified, as proved by the positive control sections coming from SNL rats.

### 5.3.3 DRG distribution of Fast Blue fluorescence

In order to confirm the distribution of FB fluorescence in the lumbar DRGs and because many sections were available, so the data from both studies was pooled.

Again, the FB fluorescence levels were higher in L3 and L4 DRGs (between around 2.4 and 3.5 % of the cells counted) than in L5 (0.7-1.2%).

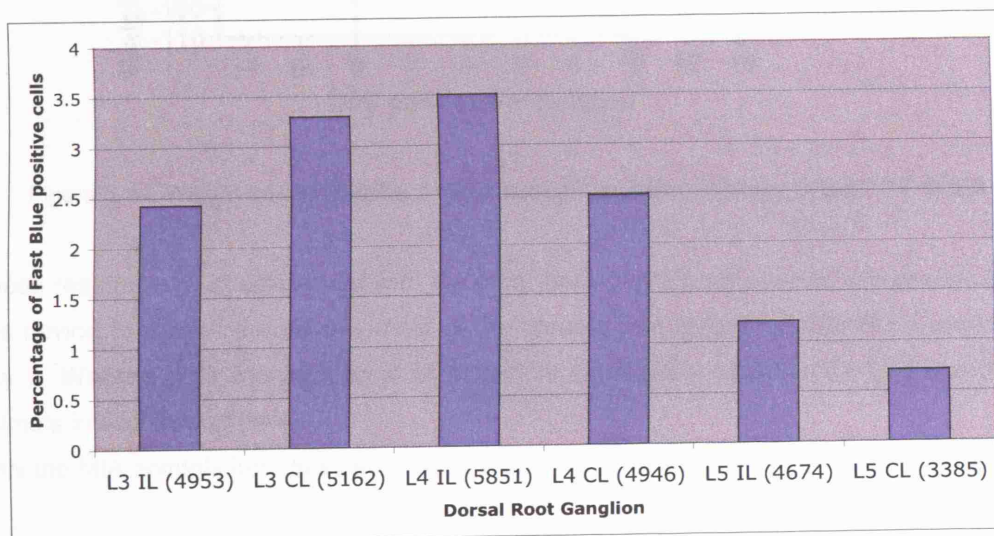


Figure 5.13 Percentage counts of Fast Blue positively labelled cells at each lumbar dorsal root ganglion. The numbers in brackets denote the total number of neuronal cells counted for each spinal level using beta-III tubulin staining.

These results were in agreement with previous findings showing that primary afferent fibres innervating the knee had their cell bodies mainly located in L3 and L4 DRGs (P. T. Salo and E. Theriault, 1997) and L4 (J. Fernihough et al., 2005).

### 5.3.4 *Alpha-2-delta-1 VGCC subunit real-time qPCR*

#### 5.3.4.1 Behaviour

Before the tissue collection for the real-time qPCR studies, animals were used to validate the use of the incapacitance tester in our model.

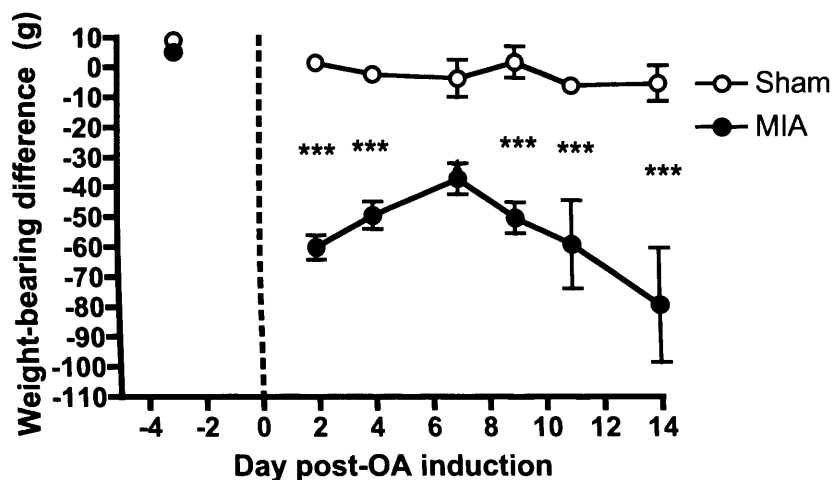


Figure 5.14. Weight-bearing results.  $n=4$  for each group. Mann-Whitney U-test, \*\*\* $P<0.001$ .

These results were in agreement with the other tests and clearly showed the usefulness of this device to assess the development of the chronic pain state following MIA injection on day 0. We can note the high level of statistical significance despite the low number of animals in each group ( $n=4$ ).

Only the MIA animals from this behavioural study were used in the real-time qPCR study.

#### 5.3.4.2 Real-time qPCR

These results are shown in Figure 5.15, comparing the mRNA levels for various receptor subunits in IL and CL DRGs for L4 and L5+L6, in both MIA (A) and SNL (B) rats.

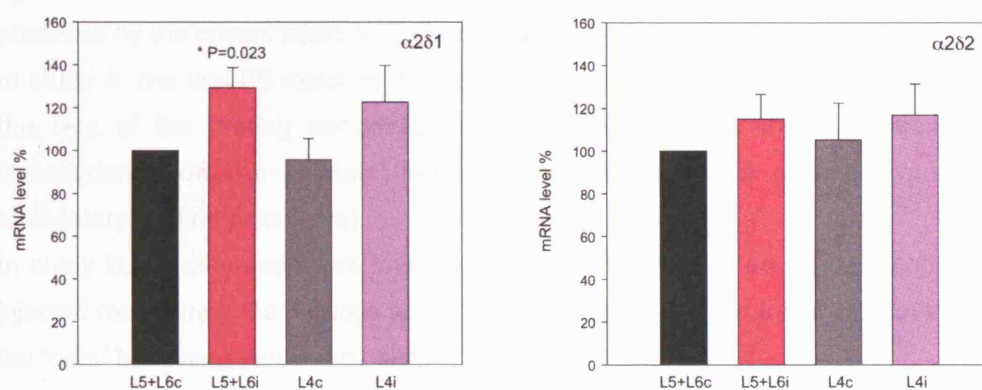
Alpha-2-delta-2 subunit mRNA levels were not statistically different in IL and CL DRGs from MIA rats, in both L4 and L5+L6, contrary to the animals that underwent SNL.

For the alpha-2-delta-1 subunit, there was a small statistically significant difference (around +25%,  $P < 0.05$ ) between its level of expression within the L5+L6 DRGs when comparing both the lesioned (IL) and control side (CL) but not within L4 DRGs.

An interesting comparison could then be made with the same tissues coming from SNL rats where the increase for alpha-2-delta-1 mRNA levels was around +300% in IL L5+L6 DRGs and around +100% in IL L4. For alpha-2-delta-2, there was a decrease in mRNA levels around -50% in IL L4 and IL L5+L6 DRGs.

The RT-qPCR study showed that there was very slight increase (+25% opposed to +300% in SNL rats) in the alpha-2-delta-1 VGCC subunit expression in the IL L4 DRG of arthritic rats 14 days post-MIA injection, potentially correlated with weak neuronal damage.

A.



B.

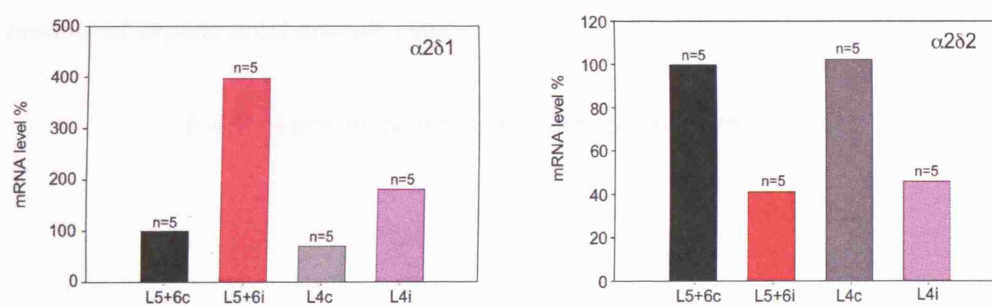


Figure 5.15. Real-time qPCR results from MIA injected rats (A) and SNL rats (B). The mRNA levels of L4c and L5+L6c were set as 100%. For the MIA study (A), n=4 in each group. Please note the different scales for mRNA levels from SNL rats (B). c: contralateral CL, I: ipsilateral IL. Data kindly provided by Dr Claudia Bauer.



## **5.4 Discussion**

### **5.4.1 *Behavioural results***

Before collecting tissues for the immunohistochemical studies, it was checked (on day 14) that the animals used were presenting the usual OA pain-like behaviour. No change was produced by the control injection in the contralateral knee joint.

In study A, the first FB injection on day -6 did not provoke any difference on day -3 and the rest of the testing period for any test, showing that the FB injection had no consequence on the animals' behaviour (mechanical and cooling hypersensitivity, ambulatory evoked pain score).

In study B, no difference was found between [FB+MIA]-injected animals and [MIA only]-injected rats, during the 14 days post-injection showing that the tracer did not interfere with the "pain" behaviour induced by MIA.

The change in protocol was made in an attempt to minimise the number of procedures undergone by the animals, to avoid any unnecessary trauma to the knee joint and to thus decrease the variability that could be added by doing two intraarticular injections instead of one.

Due to the absence of behavioural changes on the contralateral hindpaw, the use of the contralateral side as a control was even more justified, again, in a attempt to minimise the number of experimental animals used.

### **5.4.2 *Lack of neuropathic component in the model***

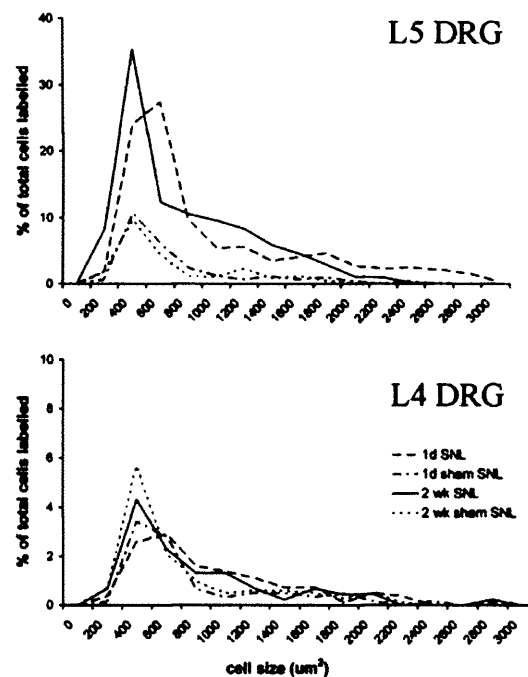


Figure 5.16. Cell size distribution of ATF-3 positive neurones expressed as percentage of the total labelled population in the L4 and L5 DRG in experimental neuropathic (L5 SNL) and sham-operated animals (P. J. Shortland et al., 2006).

Shortland et al. (2006) showed that following L5 spinal nerve transection, the rate of expression of ATF-3 in the L5 DRG (ipsilateral to the lesion) was superior to 98% and around 44% in sham operated animals (simple exposure of the nerve) on day 1, which was maintained for 14 days. In the L4 DRG, ATF-3 levels were around 11-12% in both sham and operated rats, from day 1 till day 14 (Figure 5.16).

That study showed three interesting facts. Firstly, when direct nerve damage was produced, the level of expression of ATF-3 was very high. Secondly, the influence of the surgery was important: the simple exposure of a spinal nerve could induce the expression of ATF-3 in nearly half of the cell bodies. Thirdly, the expression of ATF-3 was not confined to a single DRG in that model, although only L5 was transected.

In the MIA model, a very low percentage of ATF-3 positive cells was found in both study A (maximum of 0.12% of ATF-3 immunoreactive cells detected in IL L3 DRG) and study B (maximum of 0.18% in IL L5 DRG), which could lead to the conclusion that there was no neuropathic component 14 days after the MIA injection. Indeed, in models of neuropathy such as the L5 spinal nerve ligation model, around 35% of neurones were reported to be

ATF-3 immunoreactive two weeks post-surgery (P. J. Shortland et al., 2006), which was in agreement with my positive control sections.

Consequently, according to the immunohistochemical studies, the injection of MIA and production of the model did not cause any neuronal damage.

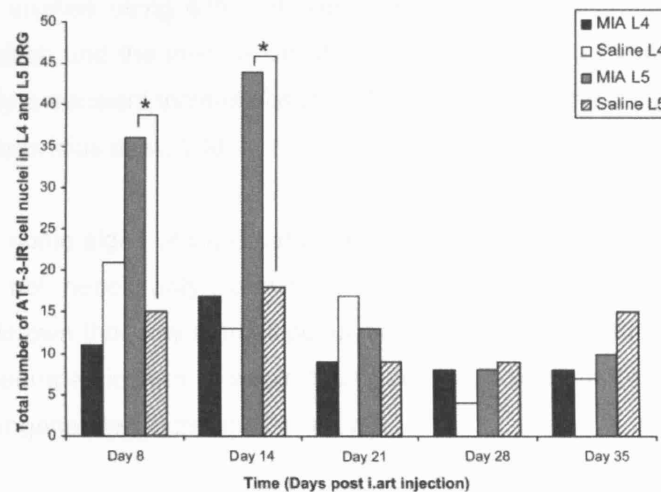


Figure 5.17. ATF-3 immunoreactivity in L4 and L5 following OA induction with MIA on day 0. Data presented as total positive cell counts ( $n=4$  per group per time point). \*  $P<0.05$  Kruskal Wallis and Mann-Whitney U-test, compared to saline controls (S. P. Ivanavicius et al., 2007).

Previously, in a similar model of OA where they used 1 mg of MIA and I used 2 mg of MIA to induce OA, Ivanavicius et al. (2007) reported higher levels of ATF-3 in ipsilateral L5 DRGs (36% on day 8 and 43% on day 14,  $P<0.05$ ) but not in ipsilateral L4 (10 and 17% respectively, non-significant difference), with levels in sham operated animals just under 10% (Figure 5.17). Those elevated levels then decreased to 15% in IL L5 and 8% in IL L4 on day 21, when the statistically significant differences levels of expression of ATF-3 disappeared, up to the last experimental day 35 following OA induction. Those results, like ours, tended to suggest that the chronic pain state was not due to a neuropathic component, while there might have been a transient low-intensity neuropathic damage caused by the injection itself to a specific knee area, the spinal nerves L4 and L5 being known to be the main branches of the sciatic nerve in the rat (F. Asato et al., 2000). Although the skin in the knee area is mainly innervated by the saphenous nerve (with dorsal horn terminals located between mid-L2 and the rostral parts of L4 (J. E. Swett and C. J. Woolf, 1985)), many other anatomical structures coming from L5 could have absorbed small amounts of MIA.

The real-time qPCR study performed by Drs Laurent Ferron and Claudia Bauer showed that there was no upregulation of alpha-2-delta-1 VGCC subunit mRNA levels in IL L4 DRG and a slight increase in L5+L6, suggesting that there was no formal evidence of any neuropathic damage induced by the MIA injection. These qPCR results were in agreement with the previous immunohistochemical studies. It is interesting to notice that consistent although small signs of neuropathy were found in the L5 DRG ipsilateral to the lesioned side by all three studies using different techniques. Again, this phenomenon might be linked to the injection and the innervation of the knee and the surrounding tissues, but it appears to be only a transient increase as ATF-3 was not expressed after day 14 post MIA injection (S. P. Ivanavicius et al., 2007).

Interestingly, had some signs of significant nerve damage been found, the pain behaviour observed would not necessarily have been caused by this neuropathic component. Indeed, it is well known that only a small population of patients undergoing a nerve trauma will suffer from neuropathic pain. It would thus have been even more difficult to interpret the pain behaviour generated in this model of OA.

Finally, other markers of neuropathy such as galanin could be used to confirm this study. The main problem with galanin is its basal constitutive expression level: the quantification of a change in expression intensity would be more arduous.

#### **5.4.3 Retrograde labelling**

Joint innervation consists of a network of myelinated and unmyelinated nerve fibres in the articular capsule with free, complex or encapsulated endings, with similar findings in tendons, ligaments, deep fascia, periosteum as well as in the synovium, conveying sensory and autonomic information, which certainly play an important role in both healthy or arthritic joint homeostasis through neurogenic mechanisms (For a review, see B. L. Kidd et al., 1990).

The main result in this study was the low number of FB marked cells. In other studies using FB or Fluoro-Gold, either the number of fluorescent marker positive neurones or the percentage of positively marked neurones were given, but the total number of cells counted was never provided, making direct comparisons impossible. I found one study which quoted that joint afferents constituted a very small proportion (<1%) of the DRGs neuronal populations (P. T. Salo et al., 2002). The rates of 2.5 to 3.5% of FB marked cells that we found in L3 and L4 were thus in agreement with that statement. Moreover, we

have to bear in mind that at the time of the injection, in both study A and study B, the (aneural) cartilage was intact. As a result, the underlying innervated subchondral bone remained intact and its nerve afferents were not in direct contact with the synovial fluid and thus could not take up FB. On the other side, the synovial capsule was innervated as well as the other components of the knee joint, and one could have expected its neurones to all be marked with FB. However, it is easy to imagine that most of the primary afferents' terminals were not actually in direct contact with the synovial fluid. Consequently, without any physical assault to the joint and to the synovium in particular, FB could well be just filtrated and be eliminated in the bloodstream. In a study with FB, the transected sciatic nerve was actually bathed by its cut end into a FB solution, which resulted in the presence of FB in the spinal cord ventral horn at the L4, L5 and L6 levels, with a peak at day 7 post-transection. FB was completely gone by day 24 (S. Mishra et al., 1999).

In a preliminary study, I had used Fluoro-Gold and compared it to FB: although a slightly higher number of neurones had incorporated FG, FB marked cells were better delimited and could be counted with a high accuracy. That was the main reason why I decided to use FB and not FG. In this study, the choice of the fluorescent marker was not that crucial, because of the low level of expression of ATF-3. The ATF-3, FB and beta-III tubulin triple-labelled cells were indeed present in even smaller numbers. It is now believed that for every case, it is important to test various markers and study the differences following their uptake by neurones in the specific model used before applying previously described standardised protocols (A. Hayashi et al., 2007).

It would also have been interesting to repeat the FB injection after the MIA injection, for example on day 7, and study the distribution of the fluorescent dye on day 14. However, this was already tested and suggested a lack of difference between MIA and sham FB-injected rats (J. Fernihough et al., 2005).

In the next chapter, I will report on neuronal responses in the dorsal horn of the spinal cord in this model of OA pain.

**Chapter 6. Spinal mechanisms and the role of NK-1 neurones in the MIA  
model of OA pain**

## 6.1 Introduction

Following the behavioural studies, it was a natural progression to study the spinal mechanisms of pain in this condition. As stated in my introduction, different primary afferent fibres convey information from the periphery to the spinal cord in response to a high intensity noxious stimulus: the thinly myelinated A-delta fibres, which mostly synapse in laminae I, II and V of the spinal cord, and the unmyelinated C fibres, which mainly terminate in laminae I and II. Neuronal populations responding to noxious peripheral stimuli are well characterised in the rat. Briefly, two main laminae (according to Rexed's classification) of the spinal cord dorsal horn are involved: the superficial lamina I neurones, mainly nociceptive-specific (NS) neurones and only responding to noxious stimuli, and the deep lamina V wide dynamic range (WDR) neurones responding to a broader types of stimuli, from the innocuous to the noxious range (L. C. Seagrove et al., 2004).

### 6.1.1 *Changes in deep WDR neuronal responses*

In a model of cancer-induced bone pain (CIBP) where MRMT-1 mammary tumour cells were injected into the tibia, Urch et al. (2003) showed that in the L4-L5 spinal segments of the dorsal horn of the spinal cord, the responses of deep WDR neurones to thermal and electrical stimuli, but not mechanical (Figure 6.1), were significantly increased in cancer-injected rats compared to sham rats. They suggested that the spinal cord had undergone a shift towards a state of hyperexcitability. They also found that superficial NS cells became responsive to wide-dynamic range stimuli, which could have been driving these neuroplastic changes via ascending and descending facilitatory pathways. Interestingly, the changes in the superficial dorsal horn neurones have not been reported in neuropathy or inflammation, implying that cancer-induced bone pain was a unique pain state (L. C. Stanfa et al., 1997; V. Chapman et al., 1998; R. Suzuki et al., 2000).

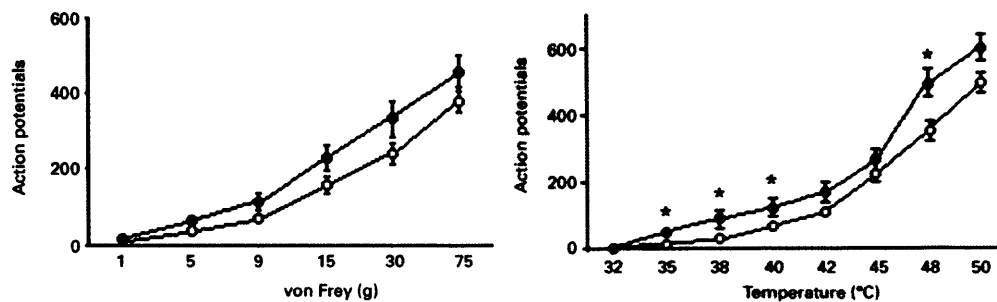


Figure 6.1. Mean responses of **deep** dorsal horn neurones in cancer-injected (closed circles) and sham-operated (open circles) rats to mechanical (von Frey hairs) and thermal stimuli (water jets set at different temperatures). Each stimulus had a 10-s duration. \* $P < 0.05$  (C. E. Urch et al., 2003).

In a Complete Freund's adjuvant (CFA)-induced model of ankle arthritis in the rat, the baseline activity of dorsal spinal cord wide-dynamic range (WDR) and nociceptive-specific (NS) neurones was increased in comparison to shams (R. Sharif Naeini et al., 2005). The application of an innocuous stimulation to the arthritic joint using von Frey hairs also elicited responses of greater amplitude and produced afterdischarge normally evoked by nociceptive stimuli.

I thus wanted to study the changes produced by the induction of OA in the arthritic rats.

### 6.1.2 Role of NK-1 receptors in this model of OA

On the basis of the study where Urch et al. found an increased excitability in superficial dorsal horn neurones in their CIBP model, investigating the role of those superficial neurones in the MIA model of OA pain appeared important.

Indeed, chronic pain conditions can be caused by tissue damage (e.g. nerve damage) or ongoing disease states (e.g. diabetes), which can cause both peripheral and central sensitisation and altered function, in primary afferent fibres and spinal cord dorsal horn neurones respectively. The dorsal horn neurones can then be part of the spinothalamic and spinoparabrachial tracts, depending on their projections. Following noxious stimulation, substance P (SP) can be released by subpopulations of the afferent neurones into the spinal cord (H. G. Schaible et al., 1990; A. W. Duggan et al., 1991). SP can then interact with its receptor, the neurokinin-1 receptor (NK-1), which is expressed by less than 5% of dorsal horn neurones (M. L. Nichols et al., 1999). However, the majority of lamina I spinothalamic and spinobrachial neurones express NK-1 (Y. Q. Ding et al., 1995; G. E. Marshall et al., 1996), suggesting that those neurones might play a role in the ascending



conduction of nociceptive information. Ablation of superficial dorsal horn spinal cord NK-1 expressing neurones by intrathecal injection of substance P (SP) conjugated with the ribosomal-inactivating neurotoxin saporin (SAP) attenuated thermal and mechanical hypersensitivity as well as nocifensive behaviour induced by intraplantar injection of capsaicin (P. W. Mantyh et al., 1997). It was also shown to reduce thermal and mechanical hypersensitivity in models of inflammatory and neuropathic pain, using both the formalin and complete Freund's adjuvant (CFA) models and the L4 and L5 spinal nerve ligation respectively (M. L. Nichols et al., 1999; R. Suzuki et al., 2002).

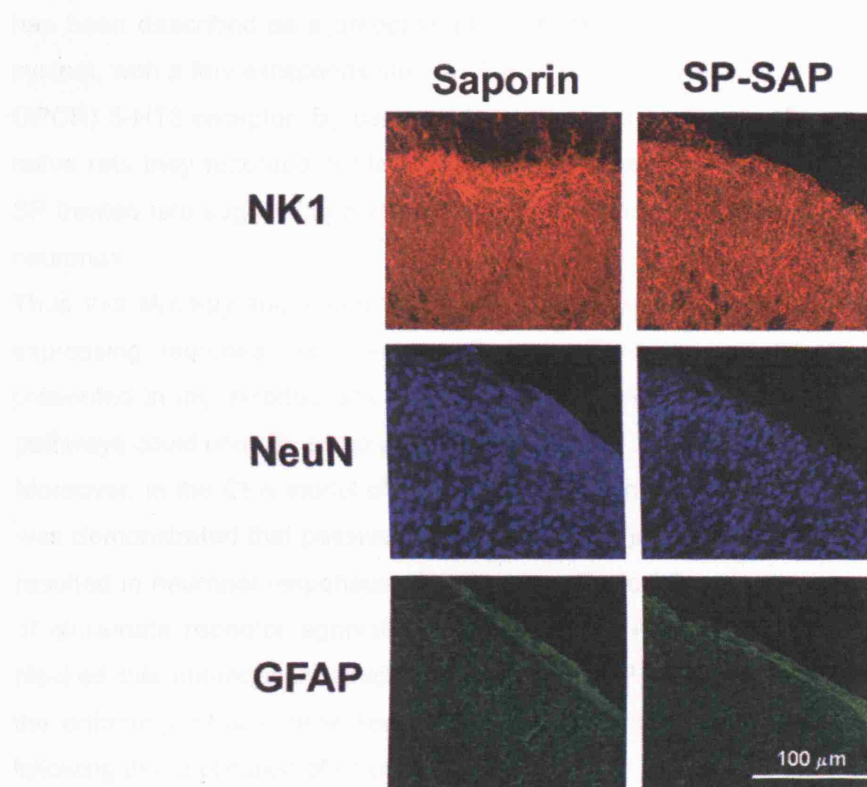


Figure 6.2. SP-SAP intrathecal injection ablated lamina I/III NK-1 receptor expressing neurones. NK1 receptor immunofluorescence in lamina I/III neurones of the lumbar spinal cord (L4–6) 28 days after treatment with SP-SAP or SAP. NeuN and GFAP staining revealed no abnormal cellular death, glial proliferation or hypertrophy (From R. Suzuki et al., 2002).

Those lamina I and/or III NK-1 neurones are also thought to be mainly nociceptive specific (NS) projecting neurones to the parabrachial area of the brain, followed by a relay in the amygdala and hypothalamus (A. J. Todd, 2002), before further projections to areas of the brainstem (PAG) that modulate descending monoaminergic pathways from the brainstem (RVM) and can regulate nociceptive processing within the spine (A. I. Basbaum and H. L. Fields, 1978; R. M. Bowker et al., 1983; J. F. Bernard et al., 1996; J. F. Bernard and R.

Bandler, 1998; M. Cui et al., 1999; Review by M. J. Millan, 2002). Following selective ablation of those neurones, Suzuki et al. (2002) not only showed an attenuation of the second phase of formalin-evoked activity and wind-up of deep dorsal horn neurones but also a decrease in mechanical and thermal coding as well as central sensitisation of such neurones. They also performed some immunohistochemical studies using C-FOS in the brainstem that demonstrated changes in the activation of serotonergic areas in the rostral ventral medulla (RVM), as well as a loss of descending control of spinal excitability following selective ablation of those neurones. Serotonin (5-hydroxytryptamine or 5-HT) has been described as a predominant inhibitory neurotransmitter in the central nervous system, with a few exceptions such as 5-HT<sub>4</sub> or the only ligand-gated ion channel (and not GPCR) 5-HT<sub>3</sub> receptor. By using ondansetron, a selective 5-HT<sub>3</sub> receptor antagonist, in naïve rats they recorded similarly reduced WDR neuronal responses to those from SP-SP treated rats suggesting a key role of this excitatory receptor in the responses of spinal neurones.

Thus this strongly suggested the existence of a spino-bulbo-spinal loop involving NK-1 expressing neurones, serotonergic cells in the RVM and spinal 5-HT<sub>3</sub> receptors (already presented in my introduction), which by contributing to medullary descending facilitation pathways could underlie some chronic pain states (F. Porreca et al., 2002).

Moreover, in the CFA model of arthritis previously quoted (R. Sharif Naeini et al., 2005), it was demonstrated that passive mobilisation of the joint from arthritic rats (and not shams) resulted in neuronal responses of greater amplitude following the iontophoretic application of glutamate receptor agonists over 10–30 min. Pre-treatment with a NK-1 antagonist blocked this potentiation, which suggested that SP was involved. Passive mobilisation of the arthritic joint also provoked NK-1 receptor internalisation, which is known to happen following the application of noxious stimuli.

For all these reasons, the second part of this chapter aimed to investigate the effects of the ablation of those NK-1 expressing neurones using SP-SAP at attenuating mechanical, thermal and ambulatory-evoked pain in the MIA-induced model of OA, as it did in other models of pain.

## **6.2 Methods**

The methods are described in the main Methods section (Chapter 2).

### **6.2.1 *In vivo electrophysiology***

Briefly, after exposing the L4-L5 segments of the spinal cord, *in vivo* electrophysiology studies were performed on anaesthetised animals, and the responses of deep WDR neurones to electrical and natural stimuli (brush, von Frey hairs and water jets at set temperatures) were recorded.

### **6.2.2 *Ablation of NK-1 expressing neurones***

Superficial NK-1 expressing neurones were also ablated using the SAP-SP toxin administered intrathecally over the spinal segments L4-L5 on day -14. Control animals only received the SAP toxin, which is not internalised and has no effect.

On day 0, animals received an intraarticular injection of MIA. Then behavioural studies using von Frey hairs, the acetone drop test and the rotarod, were conducted on those animals for 23 days post-OA induction.

## 6.3 Results

### 6.3.1 Characterisation of deep dorsal horn WDR neurones in MIA-treated rats

#### 6.3.1.1 Responses to electrical stimulation

Neurones from MIA and sham animals had similar characteristics in terms of depth and activation thresholds and although the A-beta fibre threshold tended to be reduced, being 25% of that seen in the shams, this was not significant.

	MIA	Sham
Depth ( $\mu\text{m}$ )	758 $\pm$ 41.6	621 $\pm$ 46.1
A-beta fibre threshold (mA)	0.09 $\pm$ 0.04	0.35 $\pm$ 0.28
C fibre threshold (mA)	0.98 $\pm$ 0.19	1.12 $\pm$ 0.29

Table 6.1. Characteristics of deep WDR neurones from MIA (n=9) and sham (n=8) rats. Data presented as mean  $\pm$  SEM.

Regarding their responses to peripheral electrical stimuli, the non-potentiated (NP) response and the wind-up were significantly higher in sham-treated rats. However, we can note the general trend towards an increased responsiveness in MIA-treated rats for all the modalities as illustrated in Figure 6.3. The brush response, also slightly higher in sham rats, was included on that same graph for comparison.

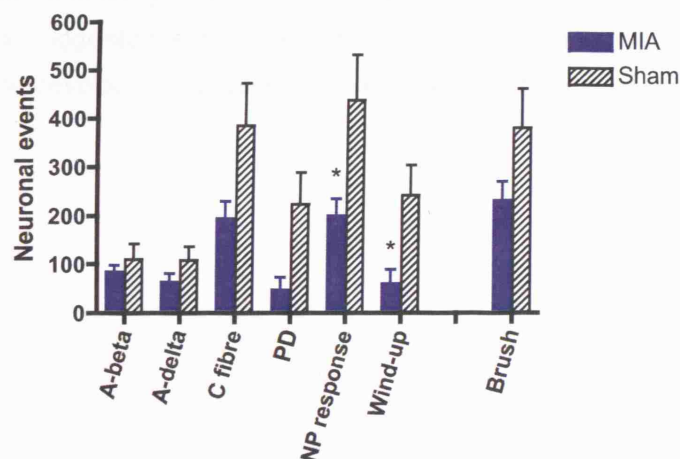


Figure 6.3. Neuronal responses to electrical tests (16 stimulations at three times the C fibre threshold) and to brushing (for 10 s) in MIA (n=9) and sham (n=8) rats. Data presented as mean  $\pm$  SEM. Student's t-test \*  $P < 0.05$ .

## 6.3.1.2 Responses to mechanical stimulation

When von Frey hairs were applied to the receptive field of the foot, there was a clear trend towards an increased responsiveness of neurones from MIA rats when compared to the shams, especially in the von Frey 6 g to 26 g range. This effect was lost with the responses to von Frey filament 60 g.

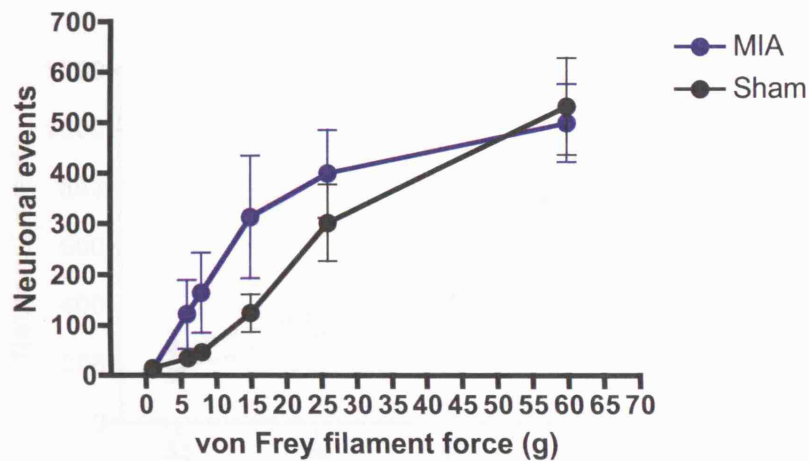


Figure 6.4. Neuronal responses to von Frey filament stimulation in MIA (n=9) and sham (n=8) rats. Data presented as mean  $\pm$  SEM.

These mechanical sensitivity data were very similar to those found in the CIBP model (Figure 6.1) and suggested a hyperexcitability state of the spinal cord, which is in agreement with the development of secondary hyperalgesia in the hind paws.

### 6.3.1.3 Responses to thermal stimulation

In response to water jets set at temperatures varying from 35 to 50 degrees Celsius, sham animals had normal gradually coded WDR neuronal responses. Neurones from MIA-treated rats followed the same tendency until 45°C but then failed to maintain their coding and had lower responses from 48 and 50°C, although that effect was not statistically significant.

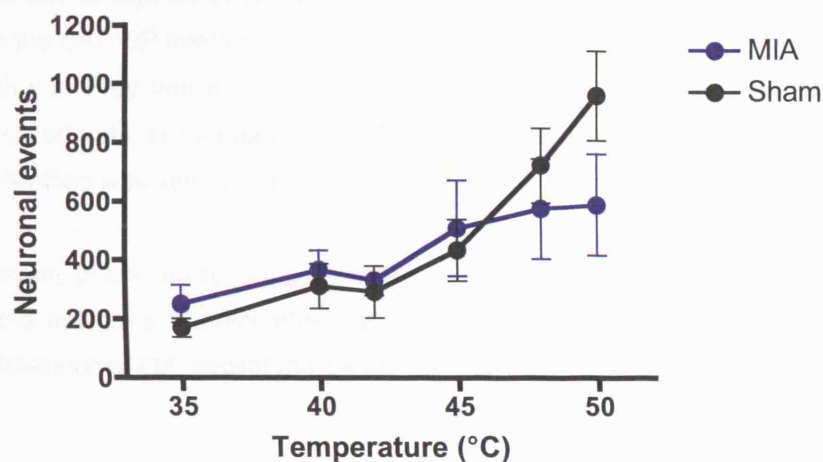


Figure 6.5 Neuronal responses to thermal stimulation in MIA and sham rats (n=8 in each group, one cell in the MIA group was lost during these tests). Data presented as mean  $\pm$  SEM.

Contrary to the CIBP model results, there was no statistically significant differences between the MIA and the sham treated groups.

Interestingly, OA patients hardly ever report heat hypersensitivity and heat hyperalgesia is usually not associated with secondary hyperalgesia, which is in agreement with the results above. Conversely, they often use warmth as an easily accessible way of decreasing OA pain.

### **6.3.2 Behavioural effects of the ablation of superficial NK-1 receptor expressing neurones**

#### **6.3.2.1 Mechanical sensitivity**

Those results are shown on Figure 6.6.

Von Frey hair 1 g (A) did not provoke any significant responses in either group.

With von Frey hair 6 g (B), there was no difference between the SAP and SAP-SP pre-treated animals, except on days 18 and 21 where a transient non-statistically significant decrease in the SAP-SP treated group was observed.

Finally, with von Frey hair 8 g (C), a clear trend towards decreased responsiveness of SAP-SP treated rats in comparison to SAP rats was present from day 2 to day 23, although this effect was very minor on days 7 and 9.

Taken together, these results suggested that the ablation of superficial NK-1 expressing neurones did not have a major effect at decreasing mechanical hypersensitivity in this model of MIA-induced OA, except maybe on day 4 and after day 14.



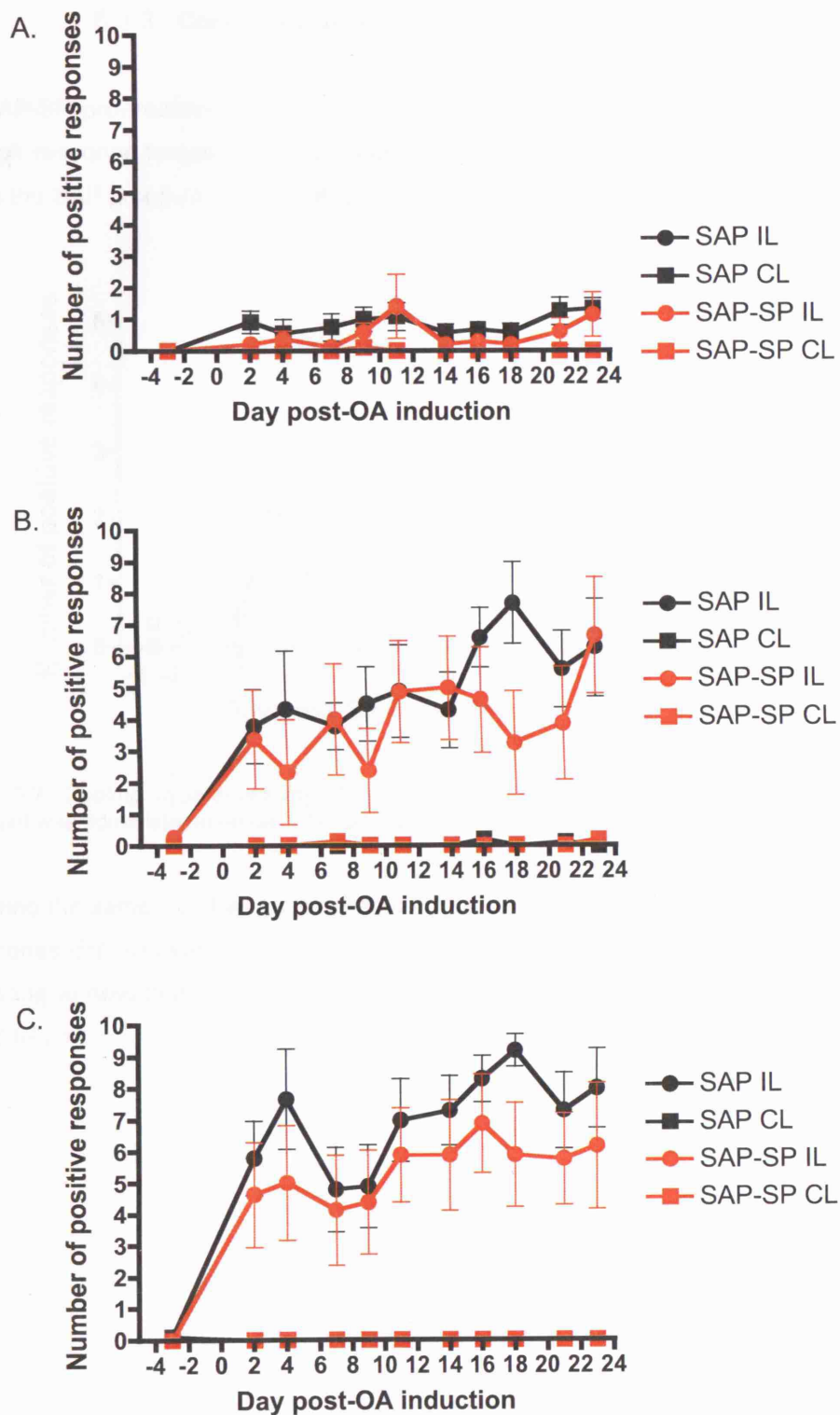


Figure 6.6. Mechanically-evoked responses to von Frey hairs 1 g (A), 6 g (B) and 8 g (C) from SAP (n=10) and SAP-SP (n=8) pre-treated rats. Pre-treatment was administered on day -14. Data presented as mean  $\pm$  SEM.



### 6.3.3 Cooling sensitivity

The SAP-SP pre-treatment did not have any significant effect on cooling hypersensitivity, although response frequencies to the acetone drop test were lower in the SAP-SP treated than in the SAP group on days 4, 18, 21 and 23 post-MIA injection.

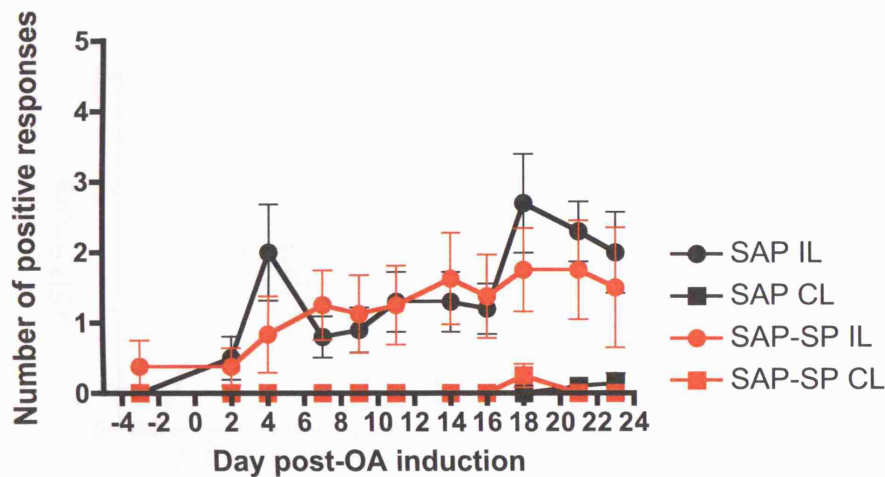


Figure 6.7. Cooling hypersensitivity of SAP (n=10) and SAP-SAP (n=8) pre-treated rats. Pre-treatment was administered on day -14. Data presented as mean  $\pm$  SEM.

Following the same trend as for mechanical hypersensitivity, the ablation of superficial NK-1 neurones did not seem to play a major role on cooling hypersensitivity. However, it was interesting to note that for both modalities, there were differences on day 4 and then from day 18 till 23.

### 6.3.4 Ambulatory evoked pain score

When scoring the ambulation-evoked pain, there was no statistically significant difference between the two groups of animals. However, there was a continuous trend towards a slightly lower score in SAP-SP treated rats between days 14 and 23, when compared to the SAP controls.

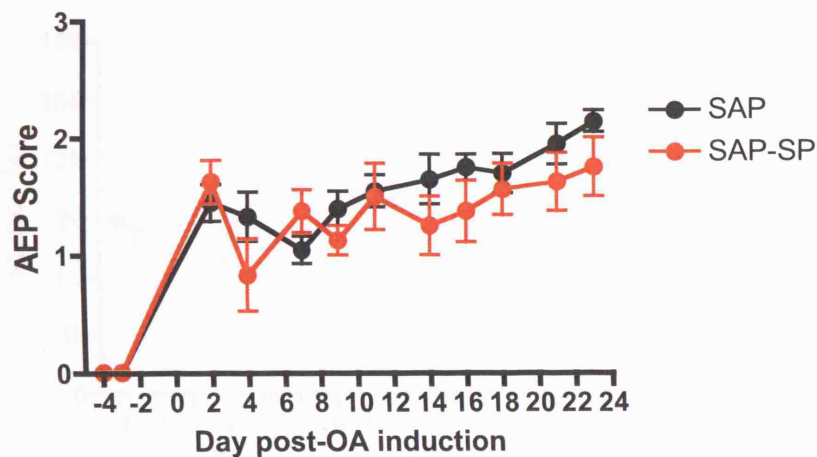


Figure 6.8. Ambulation evoked pain (AEP) score in SAP (n=10) and SAP-SP (n=8) pre-treated rats. Pre-treatment was administered on day -14. Data presented as mean  $\pm$  SEM.

The same trend towards a small effect of the ablation of NK-1 neurones was found here from day 14 onwards.

### 6.3.5 Latency to fall

The latency to fall recorded whilst scoring the ambulatory evoked pain score was not statistically significant between the two groups. Nonetheless, there was a trend towards slightly lower values in the SAP-SP group between days 14 to 18 but this effect was just transient and was absent from day 21 till day 23.

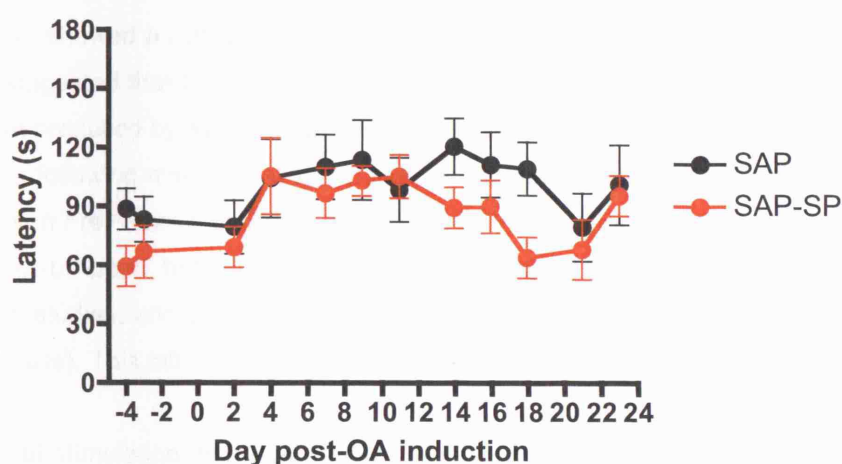


Figure 6.9. Latency to fall recordings in SAP (n=10) and SAP-SP (n=8) pre-treated rats. Pre-treatment was administered on day -14. Data presented as mean  $\pm$  SEM.

It is important to note that baseline recordings from both SAP-SP and SAP treated groups on days -4 and -3 were not significantly different. The absence of locomotive defects in those rats, which could have correlated to potential spinal cord damage caused by the intrathecal injections on day -14 could thus be confirmed for the first time.

## 6.4 Discussion

### 6.4.1 *Characterisation of neuronal responses*

Certain WDR neuronal responses to peripheral electrical stimulation were higher in the sham group than in the MIA group, especially for the non-potentiated response and wind-up that reached significance. This paradoxical finding has been reported previously in a model of shorter-term inflammation where neurones with high wind-up prior to the inflammation showed a reduced response after inflammation (L. C. Stanfa et al., 1992). It could be suggested that this involves a protective mechanism whereby high rates of firing such as that produced by wind-up can evoke inhibitory controls.

By contrast, following mechanical stimulation, there was a trend towards higher excitability elicited by von Frey hairs 6 to 26 g, which cover both the innocuous and noxious range (15 g is the cut-off-point between innocuous and noxious stimuli in the rat as seen in behavioural studies, and as also seen with *in vivo* electrophysiology when comparing NS and WDR cells). This difference was not seen with the extra-noxious von Frey filament 60 g.

After thermal stimulation, there was no difference between MIA and sham rats, although for noxious heat at 48 and 50°C (42°C is the threshold for the noxious range), there seemed to be a loss of the coding ability from those neurones. These results correlate with the lack of thermal hyperalgesia seen in OA patients.

Taken together, although there was no clear-cut neuronal hyperexcitability in the populations studied, the mechanical sensitivity results could support the development of a secondary hyperalgesia in the hind paw region, which is normally characterised by mechanical and not thermal hypersensitivity. Moreover, thermal hypersensitivity is mainly caused by C-fibre activation where mechanical hyperalgesia in the paw is likely to be mediated by A-fibres following central sensitisation, which can explain further the secondary hyperalgesia-like results studied in the hind paw.

These results could also support that of the behavioural studies from Chapter 3 validating this model of OA, which showed the presence of cooling and mechanical hypersensitivity in MIA treated animals when compared to shams.

Why did the mechanical results only approach significance?

In OA patients, as well as in our laboratory animals, joint pain is mainly evoked by mobilisation of the affected articulation. When making those electrophysiological

recordings, the joint was immobile: only the plantar part of the paw was stimulated. Unfortunately, it was not possible to make recordings following stimulation of the hindpaw and the mobilisation of the knee at the same time. Also the joint was in a stretched position that could be associated with less stimulation of the joint primary afferent fibres. Indeed, if I could have flexed the knee joint and the made some spinal cord recordings following stimulation of the paw, I might have found different results. Spinal cord neuronal recordings in response to pressure, flexion, extension, outward or inward rotation have been made in the cat (H. G. Schaible et al., 1987) although they require some specific equipment and expertise. However, such recordings would provide a great deal of information on neuronal changes directly linked to knee mobilisation and they will be the objects of attention for the next experimental plans in this model. Indeed, the results produced might be very more accentuated with differences of higher amplitude reaching statistical significance.

The other obvious issue was the fact that I made recordings from deep WDR neurones responding to stimulation of the plantar surface of the hindpaw, which is mainly innervated by the tibial nerve, a branch of the sciatic nerve itself mainly composed of the L4 and L5 spinal nerves (F. Asato et al., 2000). The knee cutaneous innervation is mainly assured by the saphenous nerve, originating mainly from L2 and L3 spinal nerves (J. E. Swett and C. J. Woolf, 1985). Moreover, the histochemical studies using Fast Blue confirmed that a healthy knee was primarily innervated by nerve primary afferents travelling through the L3 and L4 DRGs.

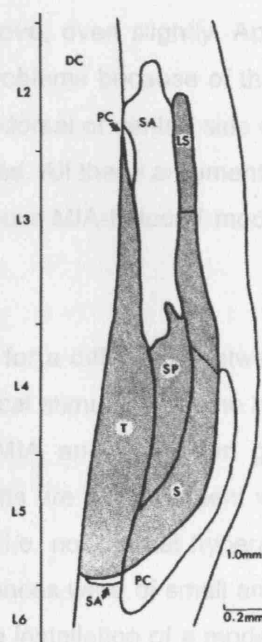


Figure 6.10. Horizontal reconstruction of labelled zones within the superficial dorsal horn from individual nerves supplying the skin of the hind leg of the rat. The ordinate represents the midline of the spinal cord. Note that the U-shaped region is composed of afferent terminations from the tibial (T), superficial peroneal (SP), sural (S) and lateral sural (LS) nerves, the four major divisions of the sciatic nerve. The saphenous (SA) and posterior cutaneous (PC) nerves, which supply the skin on the medial surface of the leg, occupy split terminal fields at the rostral and caudal edges of sciatic terminal zone (From J. E. Swett and C. J. Woolf, 1985).

However, based on the somatotopic organisation of primary afferent terminals in the superficial laminae of the dorsal horn of the spinal cord shown above, the terminal field for the tibial nerve was shown to occupy a single contiguous zone that was proportionally the largest of all nerves tested. It occupied the medial third of the superficial dorsal horn in the L4 and L5 segments with a slender rostral extension on the medial edge of the saphenous terminal field (J. E. Swett and C. J. Woolf, 1985).

As a consequence, by recording from neurones responsive to stimulation of the plantar surface of the hindpaw, although most of the recordings would have been made at the L4 and L5 levels, L3 could also have been involved, which supports the validity of the recordings I made. Indeed, recordings from WDR neurones responding to stimulation of the knee itself would not have been easy: the laminectomy would not have been problematic, as it would just have implied the removal of one extra rostral vertebra, but it is the choice of the nature of the stimuli itself that would have been difficult. In theory, one of the best stimulation of the knee would consist of a knee mobilisation, with the possibilities of a flexion or an extension, which could however destabilise the *in vivo* electrophysiology setup: in particular, it would certainly incur the loss of any isolated neurone as the spinal

cord would be highly likely to move, even slightly. Applying von Frey hairs or water jets would cause the same type of problems because of the proximity of the cord. Further, the issue of whether to stimulate the dorsal or ventral side of the knee, with the issue of having to turn over the rat would be raised. All these arguments support the stimulation of the paw as a tool to try and understand our MIA-induced model by creating a well-characterised model of OA.

In conclusion, there was a trend for a difference between deep WDR neuronal responses recorded in response to mechanical stimulation of the hindpaw and no difference at all with heat stimulation between the MIA and the sham groups. Both the thermal and the mechanical hypersensitivity results are in agreement with the usual characteristics of the area of secondary hyperalgesia, i.e. no thermal hyperalgesia but presence of mechanical hyperalgesia, although the differences were of small amplitude in response to the von Frey stimuli. These results suggest the installation of a moderate central sensitisation state. The reduction in wind-up for the MIA-treated rats does not rule that hypothesis as several different mechanisms – any that can increase neuronal responses – are likely to be involved in central sensitisation. Also, electrophysiological recordings were only made around day 14 and the extent of central sensitisation might well have varied between day 0 and day 14. Moreover, the fact that recordings were made in a zone of the cord responsive to the paw mainly, which corresponds to the terminal field of the tibial nerve which partly innervates the knee joint, without checking an input from the knee joint as well, in addition to the low number of neurones could also explain this phenomenon.

Interestingly, another electrophysiological study was performed by Dr Wahida Rahman in the laboratory, comparing both MIA and sham rats. That study included a higher number of neuronal recordings ( $n > 30$  in each group) and there was no statistically significant difference between those two groups, although the same trend was still present. However, there was a difference between those animals and the ones that I used. In my study, the animals used for electrophysiology had all been tested behaviourally on days 2, 4, 7, 9, 11 and 14 post-MIA injection, whereas in Dr Wahida Rahman's study, the behaviour was only checked once on day 14. This could have implied a loss sensitisation through decreased usage of the lesioned leg. Indeed, it was reported that in mice spontaneous models of OA, the simple addition of a new housing material in the cage (such as a small box) delayed the development of OA by a few weeks, as the mice were found to run less (unpublished data from the Conference on the preclinical models of OA, Montreal, 2006). Likewise, the

lack of regular behavioural assessment, on the rotarod in particular, could have played the same role here.

Moreover, the same model of OA pain was developed in mice, where Dr Victoria Harvey showed, after validating the pain behaviour, clear trends towards higher responses to natural stimulation in the MIA treated group, with statistically significant differences in higher range of mechanical forces, with von Frey hair 26 and 60 g in particular. A-delta, C fibre, input in response to electrical stimulation, as well as pinch were also increased, but there was no difference in A-beta or C fibres current activation thresholds.

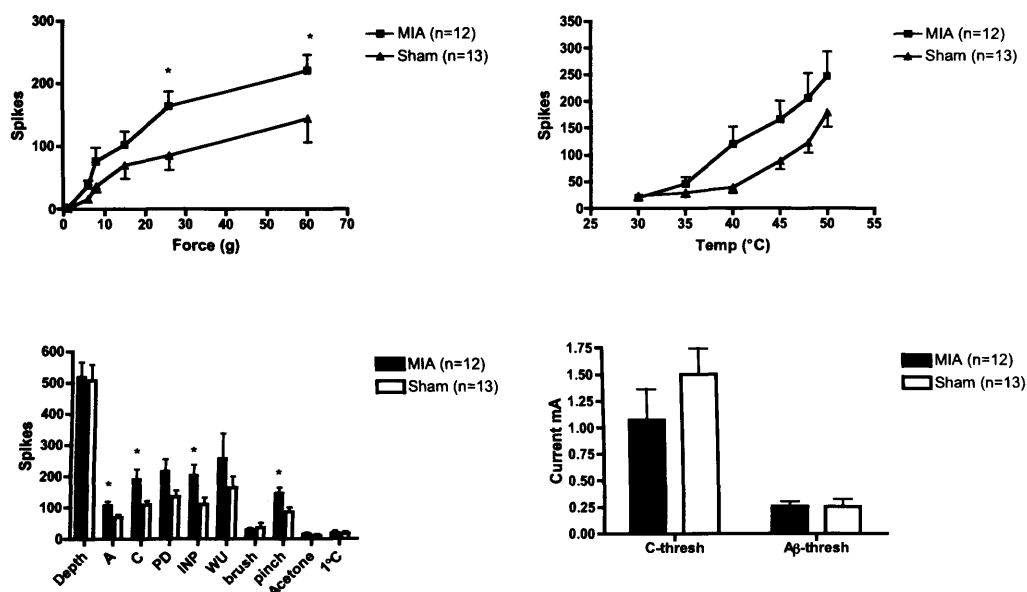


Figure 6.11. *In vivo* electrophysiological results from mouse spinal cord dorsal horn deep WDR neurones to electrical and natural peripheral stimulation of the foot, 15 to 21 days following the intraarticular injection of MIA (n=12) or saline (n=13). Note the lack of difference between current thresholds for C and A-beta fibres. Data presented as mean  $\pm$  SEM. Student's t-test for electrical tests and one-way ANOVA for natural tests, \*  $P < 0.05$ . INP: input, or non-potentiated response; WU: wind-up. Results kindly provided by Dr Victoria Harvey.

It must be noted however that the size of a mouse hindpaw is much smaller than a rat's and that the stimulation of the receptive field using the same sets of tests used in rats, could well be mobilising slightly the knee joint at the same time, which could partly explain these results.

Interestingly in that same murine model, the Hargreaves' behavioural test, investigating withdrawal latencies following the application of heat, showed no heat hypersensitivity in MIA mice when compared to shams, which was consistent with OA patients and both rat



and mouse *in vivo* electrophysiology results (in response to thermal stimulation of the hindpaw).

In the CFA-induced model of ankle monoarthritis (R. Sharif Naeini et al., 2005), higher neuronal baseline responses were recorded. However, this model is different from the MIA-model of OA as it is induced with CFA (Complete Freund's adjuvant), which is an antigen solution emulsified in mineral oil (Freund's adjuvant) that includes inactivated and dried mycobacteria, usually *Mycobacterium tuberculosis*, the pathogenic agent of tuberculosis. Freund's adjuvant is pro-inflammatory and is hence used in animals as immunopotentiator (booster of the immune system) for certain immunisations. As it is known to stimulate production of tumor necrosis factor (TNF), and thus causing a long-lasting inflammation, this model is closer to rheumatoid arthritis (RA) and more joint-deleterious than osteoarthritis, which can explain the differences.

All in all, *in vivo* electrophysiological results from spinal cord deep WDR neurones in response to stimulation of the hindpaw showed trends towards a higher excitability of those neurones in response to mechanical but not thermal stimuli from MIA-injected rats in comparison with shams. This is in agreement with the development of secondary hyperalgesia in the hind paw following central sensitisation of neurones in the spinal cord. The next study investigates the role of superficial NK-1 receptor expressing neurones in this central sensitisation state.

#### **6.4.2 Role of superficial NK-1 receptor expressing neurones in the MIA model of OA pain**

The behavioural results showed that the ablation of superficial NK-1 receptor expressing neurones caused a trend towards an attenuation of mechanical and thermal hypersensitivity in SAP-SP treated rats in comparison to SAP controls. However, statistically significant differences were never reached, up to 23 days post OA-induction, i.e. 37 days post-intrathecal injection of SAP-SP or SAP, when both OA pain behaviour and the ablation of NK-1 neurones, respectively, were well characterised. This tendency mirrors much of the neuronal data in the previous section.

Indeed, when injected intrathecally at the L4 spinal level, SAP-SP was shown to provoke a significant ablation of superficial NK-1 receptor expressing neurones (a decrease of 85% of immunoreactive cells in comparison to saline controls) between the spinal cord

segments L2 and L5, 28 days post-injection (P. W. Mantyh et al., 1997), which was confirmed by immunohistochemistry on a few rats prepared for this study. Moreover, no locomotive defects were identified through the recording of the rotarod latencies. There was thus no technical issue associated with those intrathecal injections. Concerning that matter, the use of a new Sar-SAP-SP conjugate with a slower catabolism allowing the use of slower doses, was recently investigated following concerns over non-specific damage and decreased reflex responses caused by the toxin and showed similar activity on the formalin test and formalin-induced FOS expression (R. G. Wiley et al., 2007).

*In vivo* electrophysiology was also performed on those animal populations by Dr Amy Fisher, and her results are presented below.

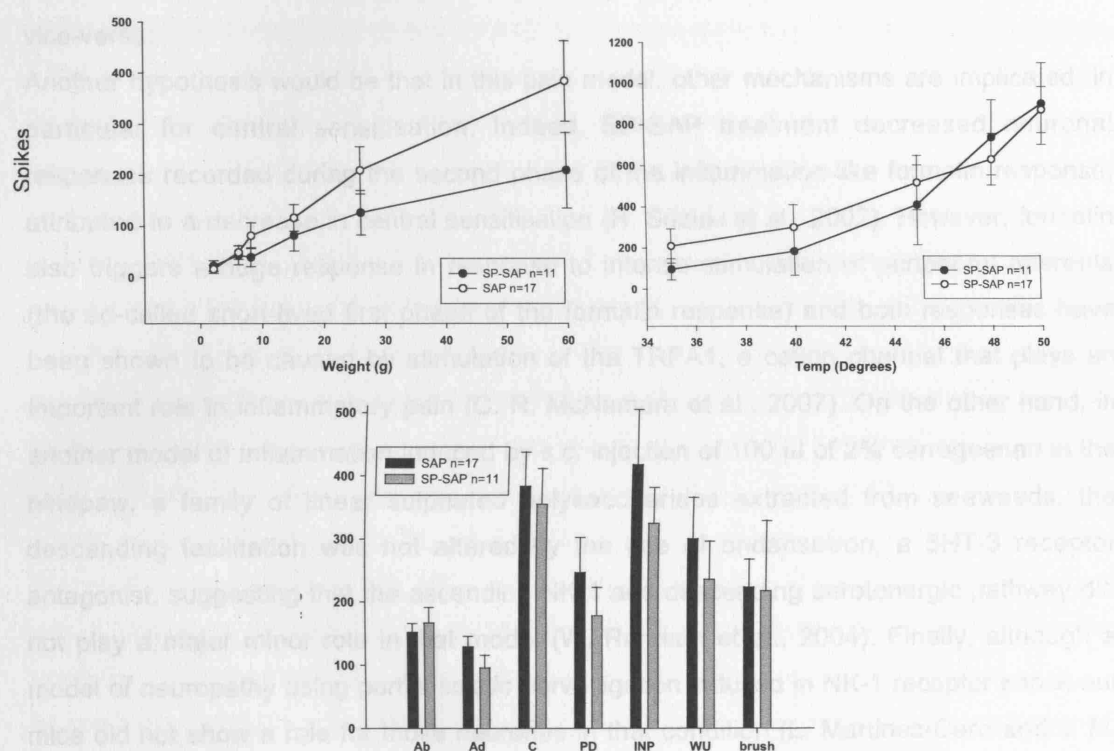


Figure 6.12. *In vivo* electrophysiological results from rat spinal cord dorsal horn deep WDR neurones to natural and peripheral stimulation of the foot, 14 to 18 days following the intraarticular injection of MIA, corresponding to 28 to 32 days post-intrathecal injections of SAP-SP (n=11) or SAP (n=17). Data presented as mean  $\pm$  SEM. INP: input or non-potentiated response; WU: wind-up. Results kindly provided by Dr Amy Fisher.

As reported previously the ablation of NK-1 expressing neurones in the lamina I region of the spinal cord reduced the ability to accurately code mechanical stimuli, as well as wind-up and caused a reduction in central sensitisation and it was likely that lamina I NK-1

receptor expressing neurones were at the origin of the spino-bulbo-spinal loop, which drove descending facilitatory controls from brainstem areas (R. Suzuki et al., 2002). Thus the mild decrease in mechanical and thermal sensitivity seen in SAP-SP rats in this MIA-induced model of OA were likely to be caused by the loss of descending facilitation, which could cause an attenuation of central hypersensitivity and central sensitisation.

However throughout this investigation, although differences occurred between SP-SAP and SAP animals, there was no statistically significant difference, neither in terms of behavioural or neuronal changes.

One explanation could be that when the behaviour or electrophysiology were performed, between 14 and 23 days post-OA injection, the resulting chronic nociceptive pain state following the inflammatory condition might have not been fully in place, and may not have necessarily modified the circuitry between the periphery, the spinal cord, the brain and vice-versa.

Another hypothesis would be that in this pain model, other mechanisms are implicated, in particular for central sensitisation. Indeed, SP-SAP treatment decreased neuronal responses recorded during the second phase of the inflammation-like formalin response, attributed to a decrease in central sensitisation (R. Suzuki et al., 2002). However, formalin also triggers a huge response in response to intense stimulation of peripheral afferents (the so-called short-lived first phase of the formalin response) and both responses have been shown to be caused by stimulation of the TRPA1, a cation channel that plays an important role in inflammatory pain (C. R. McNamara et al., 2007). On the other hand, in another model of inflammation induced by s.c. injection of 100  $\mu$ l of 2% carrageenan in the hindpaw, a family of linear sulphated polysaccharides extracted from seaweeds, the descending facilitation was not altered by the use of ondansetron, a 5HT-3 receptor antagonist, suggesting that the ascending NK-1 and descending serotonergic pathway did not play a major minor role in that model (W. Rahman et al., 2004). Finally, although a model of neuropathy using partial sciatic nerve ligation induced in NK-1 receptor knock-out mice did not show a role for those neurones in that condition (L. Martinez-Caro and J. M. Laird, 2000), the coding of deep WDR neurones to thermal and mechanical stimuli was shown to be attenuated in NK-1 knock-out mice (R. Suzuki et al., 2003). Furthermore, the neuronal and behavioural changes induced by the SNL model of neuropathy were robustly attenuated by SAP-SP pre-treatment (R. Suzuki et al., 2004), as well as the behavioural tests following SNL and three models of inflammation, formalin, carrageenan and CFA (M. L. Nichols et al., 1999). The potential role of these neurones in other more complex pain states such as the MIA-induced model of OA have not been previously studied, but my results suggest that this role is considerably less than that seen in models of nerve injury

and chemical nociception. This may reflect differences between the systems recruited by a physiological event such as tissue damage compared to the pathological state of nerve injury. As stated in my introduction as well, the SP receptor expressing neurones only represent 5 to 10% of the total number of lamina I neurones, and although most of them project to higher areas of the brain involved in nociceptive signalling, other pathways are likely to be involved as well.

The relative roles of NK-1 receptor expressing neurones and GABAergic spinal inhibitory systems in the development of chronic pain states have also been investigated as they could both play different roles in chronic pain states. In the CFA model of tibiotarsal monoarthritis, the use of SAP-SP coupled with baclofen, a selective GABA<sub>B</sub> receptor antagonist, showed that both NK-1 and GABA<sub>B</sub> receptors participated in the development of "secondary hyperalgesia" that had previously been shown to be accompanied by an imbalance between inhibitory and excitatory neurotransmission at the spinal cord level, although the decrease in GABA<sub>B</sub> inhibition seemed to play a more important role than the facilitation mediated by NK-1 neurones (A. R. Castro et al., 2005, 2006). GABA (gamma-amino butyric acid) is one of the main neuromediators of local endogenous inhibition and is thus a very important actor in intrinsic spinal inhibitory systems, which following a noxious stimulation, participates, in addition to supraspinal pathways and their resulting descending inhibitory and facilitatory counterparts, to the overall spinal cord activity balance. An *in vitro* study showed that superficial NK-1 receptor expressing neurones in the spinal cord were under strong GABAergic control, as the use of bicuculline, a selective GABA<sub>A</sub> receptor antagonist, revealed that those lamina I NK-1 expressing cells could receive a novel NMDA-dependent A-beta mediated input following DRG stimulation (C. Torsney and A. B. MacDermott, 2006). Rahman et al. (2007) then demonstrated that despite the loss of GABAergic inhibitory controls after SP-SAP treatment, the net effect was a decrease in spinal cord excitability, implying that activation of NK-1 receptor expressing neurones following noxious stimulation predominantly drove facilitation.

As we can see, different systems are certainly involved, and the final balance between inhibitions and excitations is not a simple equation.

Moreover, the simultaneous hypoesthesia and allodynia, with paradoxical decrease in sensation and increased pain thresholds were also recorded in OA patients and may well be the result of both peripheral and central alterations in neuronal responsiveness to mechanical stimulation and could suggest the activation of a descending inhibitory system (J. A. Hendiani et al., 2003). Indeed, these findings are reminiscent of the neuronal data that I report, in that shifts in both directions in responsivity were seen.

### **6.4.3 *Role of central and peripheral sensitisation in this model of OA pain?***

The findings from the electrophysiological study suggest that central sensitisation is taking place, which could help to explain the development of secondary hyperalgesia in the paw region. The ablation of NK-1 expressing neurones only produced a trend towards attenuation of the painful behaviour, which suggests that at that time point (day 14 post MIA), those specific neurones do not play a major role in the nociceptive modalities of this model of OA pain and inflammation.

The small differences seen could be explained by the previous hypotheses as well as, for example, the fact that in quadrupedal animals, rats in particular, a guarding, avoiding, or resting behaviour for the lesioned leg could slow down the development of central sensitisation. Indeed, contrary to humans who cannot avoid putting some strain on a knee joint (unless using crutches), rats can freely move around with only 3 legs. Dogs and cats can also live normally following the amputation of one limb. As a result, the so-called central sensitisation might be avoided by this guarding behaviour of the injured limb. The fact that MIA and sham rats have the same weight curves, as shown in Chapter 3 on the model validation, was also indicative of this non-disabling pain state, as the food intake, which involves some exercise in the cage, was not impaired. OA patients could also maybe avoid the development of central sensitisation by learning what kind of exercise or movement is likely to provoke pain.

Another theory to better understand OA pain mechanisms is the fact that peripheral sensitisation could play a bigger role than central sensitisation, as OA pain normally disappears following a joint replacement (provided no iatrogenic neuropathic pain is incurred by the surgery itself...), which would complement very well the previous discussion of the data. Indeed, it could easily be assumed that there is a constant ongoing background pain that could be worse at certain times of the day, for example in the morning. However, that pain would be very weak compared to movement-provoked pain or inflammatory pain caused by excessive exercise for example. In that context, despite the presence of this peripheral sensitisation state where movement can be painful, patients could avoid the development of central sensitisation by acting on the main source of pain, i.e. movement. In neuropathic pain for example, this avoidance behaviour would not be possible as the source of the damage would be present 24 hours a day, and would then imply changes in the spino-bulbo-spinal loop regulating that state.

The peripheral drive due peripheral sensitisation could thus well play a more important role than central sensitisation in this OA pain model.

**Chapter 7. Effects of AS006, a novel peripheral opioid receptor agonist,  
in the MIA model of OA pain**

## 7.1 Introduction

As the MIA model of OA has been successfully used to test the effects of various existing analgesic agents such as morphine and gabapentin, it was natural to try and test new potential therapeutic drugs. Thus it was decided to evaluate the effects of AS006, a novel opioid recently developed by Sanochemia and presented as having a restricted peripheral action compared to other centrally penetrating opioids, on the MIA model of OA pain.

### 7.1.1 *Opioids side-effects*

Indeed, central and peripheral side-effects of opioids are well characterised (C. E. Inturrisi, 2002; A. H. Dickenson and B. Kieffer, 2005).

#### 7.1.1.1 Central side-effects

The central side effects of opioids include nausea and vomiting probably by following the activation of a large numbers of opioid receptors in regions such as the solitary tract and adjacent areas. More importantly, respiratory depression due to the reduced sensitivity of the respiratory centres in the brainstem to  $pCO_2$  is the most common cause of death from overdose with street use of opiates. Sedation and drowsiness are other undesirable effects in analgesia.

While psychological dependence does not seem to occur to any great extent in the presence of pain, physical dependence through opioids actions can occur and require careful cessation of the opioid therapy. A peripherally restricted drug would avoid these problems.

#### 7.1.1.2 Peripheral side-effects

Other side-effects of opiates are caused by their actions on peripheral opiate receptors outside the CNS, and those would still be present when using a peripherally acting opioid. These include the main concern of patients, constipation, which is a result of a maintained contraction of the smooth muscle of the gut that reduces motility. Diminished propulsion, in addition to a reduction of secretion in the gut, also underlie an antidiarrhoeal effect. Urinary

retention, multifocal myoclonus seen at high doses, decrease in immune function are other side-effects.

### 7.1.2 AS006

*All the following information on AS006 is publicly available data and was taken from Sanochemia's Corporate profile & strategy presentation, October 2006, [www.sanochemia.at](http://www.sanochemia.at).*

AS006 is AlcaSynn's current lead product. It is a 6-Aminomorphinan derivative, patent-protected until 2021. AS006 is a mu-opioid receptor (MOR) agonist with a peripheral mechanism of action, and is due to enter Phase I clinical studies in 2008.

#### 7.1.2.1 AS006 properties

"AS006 possesses a strong yet selective effect on mu-opioid receptors (MOR), which are primarily responsible for the modulation of pain in the central and peripheral nervous system. Binding an opioid molecule to a receptor restricts the transmission of pain signals and hence the sensation of pain.

In contrast to the opioids currently in use (e.g. Fentanyl), AS006 only binds to opioid receptors present in the peripheral nervous system, because it cannot pass the blood-brain barrier. This in turn means that the side-effects usually associated with opioid pain-killers such as respiratory depression, sedative effects and addiction are avoided.

Previous comprehensive preclinical studies have established the considerable advantages of this product: AS006 shows strong yet selective bindings to MORs and has been shown to possess outstanding properties in terms of safety, toxicity and side-effects, and AS006 appears to be suitable for use in all established application forms, which in turn opens up the full spectrum of possible pain indications.

#### 7.1.2.2 AS006 and its applications

The pharmacological and chemical properties of the API (active pharmaceutical ingredient) AS006 opens up a series of possible applications in all key pain-related indications in which centrally-acting opioids are currently in use. Based on the knowledge acquired to date, there is no restriction in terms of the application forms that may be used.



Despite their side-effects, opioids remain the undisputed gold standard in the treatment of moderate to severe pain. However, professionals in the field unanimously agree that there is an acute need for innovation in this field (Analgesia: Global Markets and Trends, March 2000): 'The greatest need of the severer pain market is for a product with the analgesic power of morphine but without its addictive and other side-effects' AS006 fits this profile."

Note: It might have been more appropriate to use the term 'nociception' instead of 'pain'.

The purpose of this study was to investigate the acute effects of AS006 in this model.

## 7.2 Methods

Following a short initial pilot study to establish the appropriate dose-range (data not shown), this study was done in phases, corresponding to 2 parts A and B investigating the effects of different doses.

Animals were tested in batches of 8 rats maximum.

For each batch of rats, the following testing schedule applied:

**Day -4:** Training to familiarise rats with testing procedures and to eliminate poor rotarod performers (i.e. <30 sec. endurance), followed by baseline behavioural testing.

**Day 0:** Induction of OA through intraarticular injection of MIA

**Days 3, 7 and 10:** Behavioural testing

**Day 14:** Chronic pain state in place: behavioural testing followed by **drug treatment** by s.c. injection, then behavioural testing at 30, 120 and 240 minutes.

### 7.2.1 Pain assessment

The behavioural tests were previously described in chapter 2 (Methods), including this time the extensive use of the incapacitance tester, in addition to von Frey (VF) hairs, the acetone drop test and the rotarod.

The testing sequence for each time-point (baseline and post-drug) was as follows:

Acetone 1 – VF 1 – Acetone 2 – VF 6 – Acetone 3 – VF 8 – Acetone 4 – Acetone 5 -  
Rotarod – Incapacitance tester.

Because of the high number of rats per batch (8 rats), the rats were tested in a staggered manner in time: 4 rats would be tested and injected with their treatment, whereas the other 4 rats would receive their treatment 30' later in order to have a more precise picture of the treatments' effects through a shorter testing window.

### 7.2.2 Drug preparation and administration

Following the results of the pilot study aiming at selecting the adequate doses, the doses to be used in part A were decided, which tested one of 5 treatments comprising 3 doses of AS006, morphine as positive control and a control saline treated group.

After examination of the results of part A, part B was added and comprised 3 treatments comprising of 2 lower doses of AS006 or saline.

Part A	Part B
Saline 1 ml/kg	Saline 1 ml/kg
AS006 25 µg/kg	AS006 6.25 µg/kg
AS006 50 µg/kg	AS006 12.5 µg/kg
AS006 100 µg/kg	
Morphine 3 mg/kg	

Table 7.1. The different treatments tested in the AS006 study.

AS006 was prepared by dissolving an accurately weighed amount of the dry powder in sterile saline 0.9% to obtain a concentration of 1 mg/ml in terms of base\*, which was used as the stock solution and was retained in the refrigerator (ca 4°C) for a maximum of 7 days when not in use. Fresh stock solutions were prepared as needed and the required concentrations were made by serial dilution of the stock solution on the day of testing.

\*The doses of AS006 are expressed in terms of base (conversion factor = 1.387). For example, the 1 mg/ml base solution would contain 1.387 mg/ml of AS006 as supplied (µg/kg: micrograms per kilogram).

The reference drug was morphine hydrochloride (obtained from Thornton and Ross, batch BN VR 76), which was dissolved in 0.9% saline solution.

Compounds were administered subcutaneously (s.c.) into the loose skin at the back of the neck in a dose-volume of 1 ml/kg body weight.

Vehicle control animals received an equivalent dose-volume of sterile saline (1 ml/kg).

These injections were performed in randomised and blinded manner.

### **7.2.3 Body weight**

Body weight was monitored and recorded on days -4, 0 (day of OA induction), 3, 7, 10 and 14.

### **7.2.4 Statistical analysis**

Statistical analyses were confined to the main study. Mean values  $\pm$  SEM for each behavioural parameter were determined for each treatment group at each time-point. Mann-Whitney U-tests were used to compare the control group (saline) with each of the treatment groups (various doses of AS006 and Morphine) for non-parametric data from the mechanical and cooling hypersensitivity tests as well as the rotarod data. An analysis of variance for repeated measures was applied to the weight-bearing data (2-way ANOVA, Prism 4, GraphPad software, Inc, followed by Bonferroni's post-test comparisons with the vehicle control group as appropriate). Significance level was set at  $P < 0.05$ .

## **7.3 Results**

### **7.3.1 Part A of the AS006 study**

These results are provided in Figure 7.1 and Figure 7.2.

No difference between groups before the treatment on day 14 was present. I am therefore confining comments to the responses on day 14, i.e. the day when AS006 was injected.

For VF and Acetone tests, the contralateral responses were very low and were not different between groups, hence I only plotted and studied the differences between IL responses (cf. bar graphs Figure 7.1).

#### **7.3.1.1 Mechanical sensitivity**

With VF 1 g, there was no difference between the different groups.

With VF 6 g, there was no difference between groups except at 120' post-treatment, between the control and the 100 µg/kg groups.

However with VF 8 g, there was a difference between the control group and all the treatment groups (AS006 25, 50 and 100 µg/kg and morphine 2 mg/kg) at 30' post-injection. At 120', morphine lost its effects but the difference between the groups treated with the different doses of AS006 and the control group remained. At 240' post-injection: no treatment effect was detectable and there was no difference between the different groups. Thus AS006 produced a reduction in mechanical hypersensitivity produced by MIA.

#### 7.3.1.2 Cooling sensitivity

At 30', acetone responses were abolished by all the treatments (AS006 and morphine) except for the lower 25 µg/kg dose of AS006.

At 120', there were significant effects for all 3 doses of AS006 but not for morphine.

At 240', there was no significant effect of any treatment although the doses of 50 and 100 µg/kg tended to produce a reduced response.

#### 7.3.1.3 Ambulatory-evoked pain score and latency to fall

At 30' post-injection, the latency to fall in the treated groups was comparable to the one in the control group, indicating no effect of AS006.

At 120', the rotarod latency increased significantly in the 25 and 50 µg/kg AS006 groups, and at 240' in the 25, 50 and 100 µg/kg AS006 groups, but not in the morphine treated groups.

The increased latency indicate that AS006 enables the rats to perform better and moreover, the absence of any shortening in the latency suggests that the drug does not have a sedative effect, even at 100 µg/kg. This verifies the lack of meaningful central penetration even at this highest dose.

The ambulatory pain score was reduced 30' post-injection for the morphine and the highest dose AS006 (100 µg/kg) groups.

At 120', all the groups AS006 treated groups had a decreased score relative to the controls, but morphine had lost its effects.

At 240', a residual effect was seen, reaching statistical significance in the 25 µg/kg AS006-treated group.

We can thus conclude that AS006 is effective at relieving nociception between 30 and 120' post-injection with relief tending to extend to 240 minutes. The effect appeared to be dose-related with the maximal dose of 100 µg/kg having the greatest and most long-lasting effects.

#### 7.3.1.4 Weight-bearing

On day 14, there was a slight difference in the baseline weight-bearing measures between the control and the lowest AS006 dose of 25 µg/kg groups.

At 30' post-injection, only the morphine-treated showed an improved weight-bearing.

At 120', weight-bearing was significantly improved in the 25 and 100 µg/kg AS006 groups.

At 240', there was no effect remaining for any treatment group.

Again, these results strongly suggest that AS006 is effective at decreasing nociceptive behaviour, especially at the dose of 100 µg/kg, with an effect up to 2h post injection.

#### 7.3.1.5 Weight gain

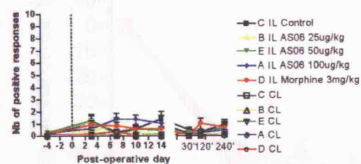
No difference was observed between groups showing that the drug-induced changes were not incidental to body weight changes.

#### 7.3.1.6 Conclusion of part A

In view of the marked effect of AS006, even at the lowest dose of 25 µg/kg, it was decided to investigate the effects of the lower doses of 12.5 and 6.25 µg/kg.

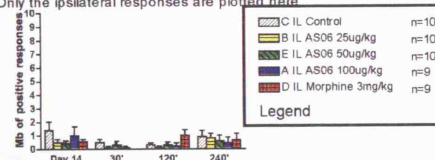
## AS006 Results

### A. Von Frey 1g

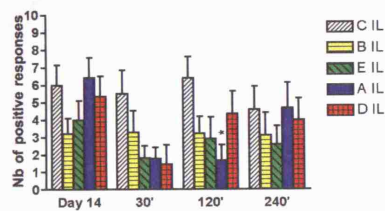
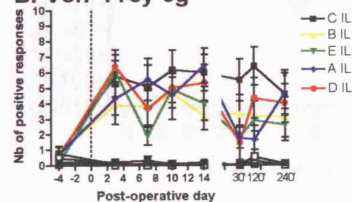


Bargraphs of day 14 responses: baseline, 30, 120 and 240min post-injection.

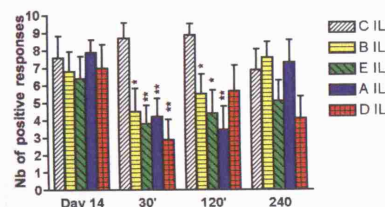
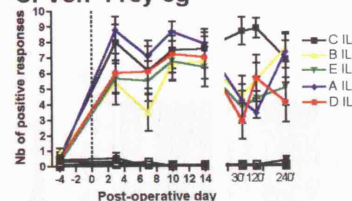
Only the ipsilateral responses are plotted here



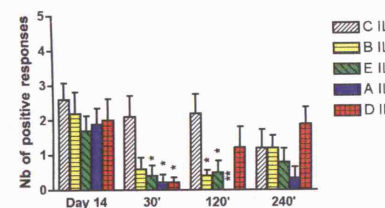
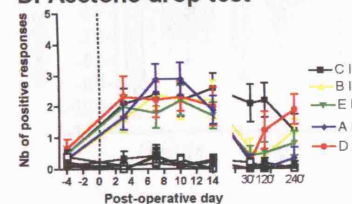
### B. Von Frey 6g



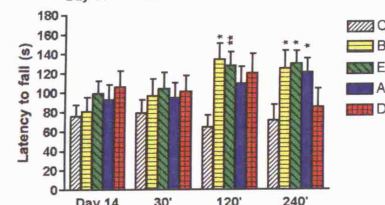
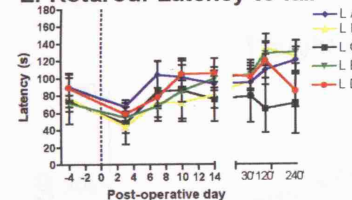
### C. Von Frey 8g



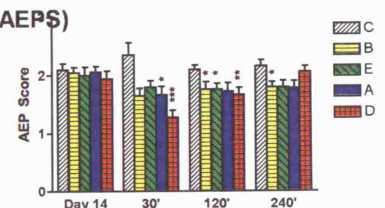
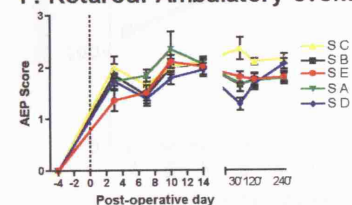
### D. Acetone drop test



### E. Rotarod: Latency to fall



### F. Rotarod: Ambulatory-evoked pain score (AEPs)



IL: ipsilateral to OA-induced side, CL: contralateral

Mann-Whitney test, comparisons with control group  
\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

Figure 7.1. Part A: Mechanical and cooling sensitivity, and rotarod results following OA-induction (day 0) and drug administration (day 14). Data presented as Mean  $\pm$  SEM, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

## AS006

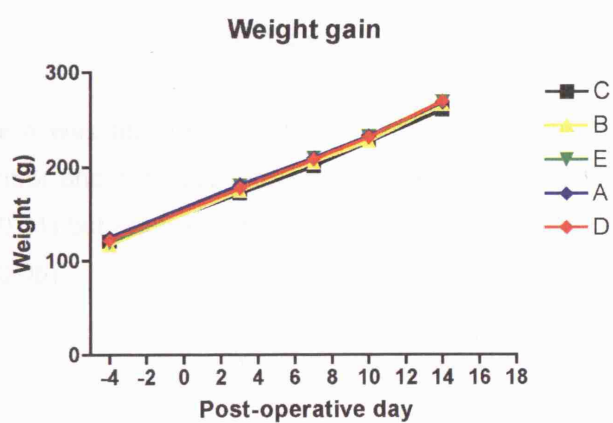
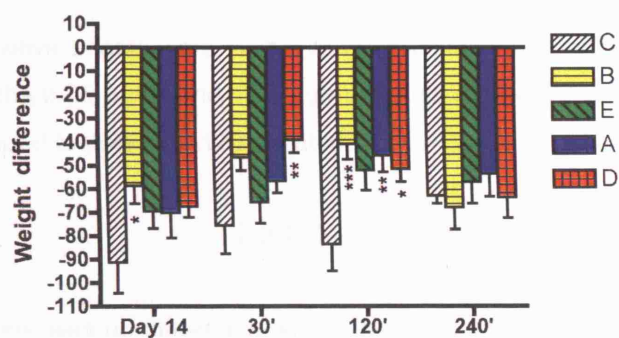
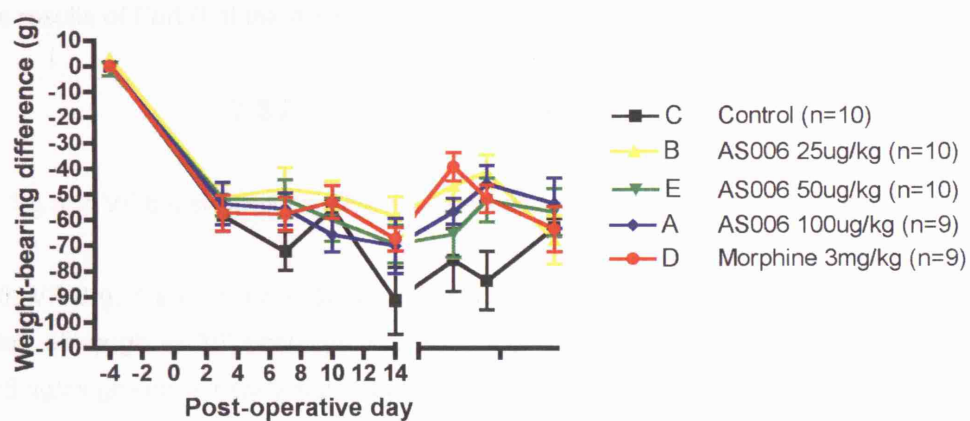


Figure 7.2. Part A: Weight-bearing and weight gain following OA-induction (day 0) and drug administration (day 14). Data presented as Mean  $\pm$  SEM, \*  $P < 0.05$ , \*\*  $P < 0.01$ .

### 7.3.2 *Part B of the AS006 study*

The results of Part B of the AS006 study are provided on Figure 7.3 and Figure 7.4.

#### 7.3.2.1 Mechanical sensitivity

VF 1 g and VF 6 g showed no difference between the different treatments groups.

With VF 8 g, there was no statistically significant difference between groups at any time-point, although at 30' post-injection, the difference between the control and the AS006 6.25 µg/ml groups is nearly significant ( $p=0.0587$ ).

#### 7.3.2.2 Cooling sensitivity

Positive effects were visible at 30' post-injection, with a significant reduction in responses in the 6.25 µg/ml and 12.5 µg/ml groups ( $p=0.0520$  and  $p=0.0209$  respectively); the effects tended to persist to 120 minutes.

#### 7.3.2.3 Rotarod

There was no difference, either in terms of latency to fall or in the ambulatory-evoked pain score of AS006 treated groups, compared with the control group.

#### 7.3.2.4 Weight bearing

There was an effect at 30' post-injection, reaching statistical significance between the control and the 6.25 µg/ml groups (average difference of 37.26 g, 95% CI [5.46-69.05],  $p<0.01$ ) but not with the 12.5 µg/ml group (average difference of 25.16 g, CI [-6.64-56.95],  $p>0.05$ ).

#### 7.3.2.5 Weight gain

All groups consisted of rats in the same weight range again confirming that the drug-induced changes are not incidental to body weight changes.



### 7.3.2.6 Conclusion of part B

There was a slight effect of AS006 at decreasing the acetone responses at 30' following the higher dose of 12.5  $\mu\text{g/kg}$ .

There is also a statistically significant effect of AS006 6.25  $\mu\text{g/kg}$  at 30' in terms of weight-bearing distribution

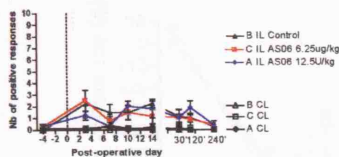
All the other tests, in particular the latency before falling from the rotarod apparatus remained unchanged by the treatments.

All in all, these results confirm results from the first part of the main study that AS006 can reduce dramatically the responses to the acetone test, even at doses as low as 12.5  $\mu\text{g/kg}$ , although this effect is short-lived compared to higher doses.

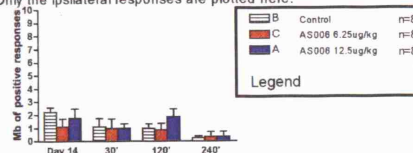
It was observed that some animals looked calm and immobile just minutes after the injections of the low doses of AS006. However, this overt clinical sign should not necessarily be interpreted as sedation since it was readily reversible by disturbing the animals and clearly did not last longer than 30 minutes after the injection. Thus, when animals were tested on the rotarod (30' time-point), they did not exhibit any difference in their ability not to fall off the rod (rotarod latency).

## AS006 batches 7, 8 and 9, Low doses

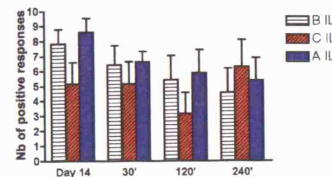
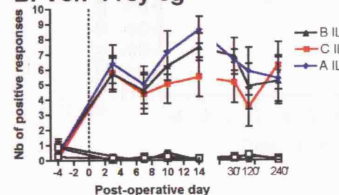
## A. Von Frey 1g



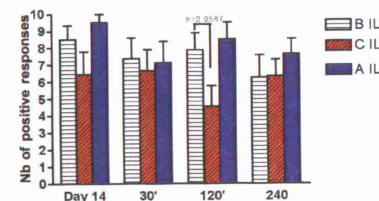
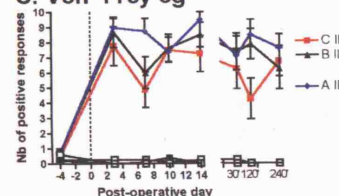
Bar graphs of day 14 responses: baseline, 30, 120 and 240min post-injection. Only the ipsilateral responses are plotted here.



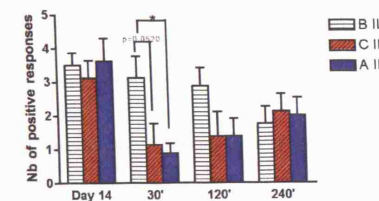
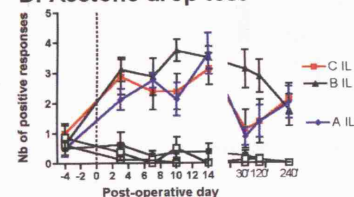
## B. Von Frey 6g



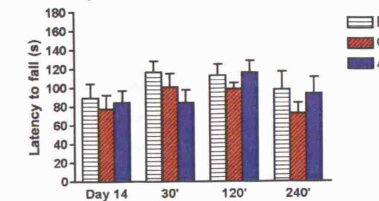
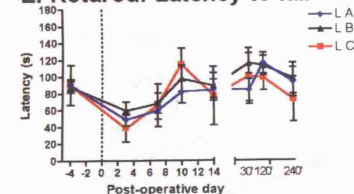
## C. Von Frey 8g



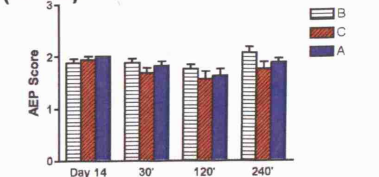
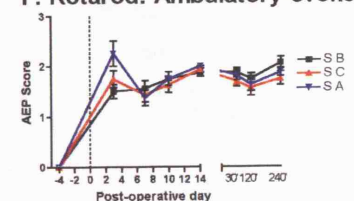
## D. Acetone drop test



## E. Rotarod: Latency to fall



## F. Rotarod: Ambulatory-evoked pain score (AEPs)

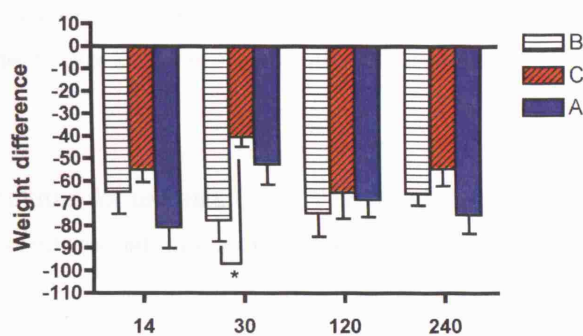
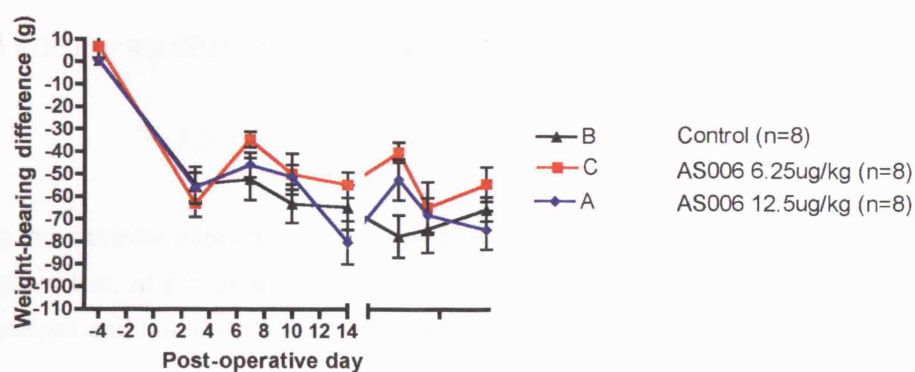


IL: ipsilateral to OA-induced side, CL: contralateral

Mann-Whitney test, comparisons with control group  
\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

Figure 7.3. Part B: Mechanical and cooling sensitivity, and rotarod results following OA-induction (day 0) and drug administration (day 14). Data presented as Mean  $\pm$  SEM, \*  $P < 0.05$ .

## AS006 batches 7-8-9, Low doses



2-way ANOVA, followed by Bonferroni post-tests, comparisons with control group B, \*  $p < 0.05$

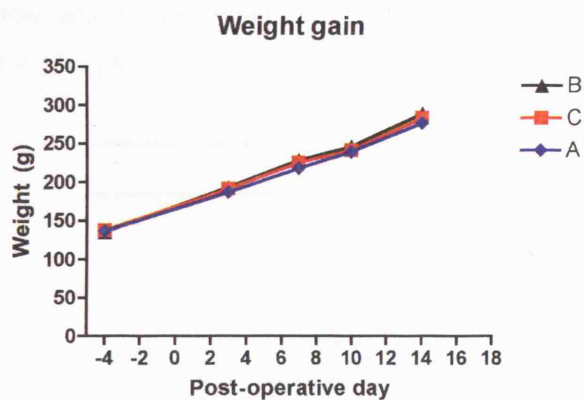


Figure 7.4. Part B: Weight-bearing and weight gain following OA-induction (day 0) and drug administration (day 14). Data presented as Mean  $\pm$  SEM, \*  $P < 0.05$ .

### 7.3.3 ED50 Calculation

AS006's ED50 for the different modalities were calculated.

#### 7.3.3.1 First method

By using the maximal effect obtained for each of the concentrations used (using the results from a given test, at any time-point) the responses were plotted as a function of the drug concentrations and realised a linear regression (Figure 7.5).

Following a linear regression, both the minimal and the maximal responses at 6.25 and 100 µg/kg respectively could be graphically determined.

Having the amplitude of the 100% response, the 50% effect and the corresponding ED50 could be found.

The ED50 was calculated for all tests (VF6, VF8, Acetone, Rotarod score, Incapacitance tester) but not for the rotarod latency as the effect of AS006 on this test is not very marked (small slope).

Finally, all ED50s values were averaged in order to have AS006 ED50 as detailed in the table below, which gave an average AS006 ED50 for all tests (except for Rotarod latency) of  $50.5 \pm 2.74$  µg/kg.

Test	ED50 (µg/kg)
VF 6 g	55
VF 8 g	47.5
Acetone	50
Rotarod Latency	N/A
Rotarod Score	50
Incapacitance	50
<b>Average</b>	50.5
<b>SD</b>	2.74

Table 7.2. AS006 ED50 based on maximum effect.

## ED50 determination

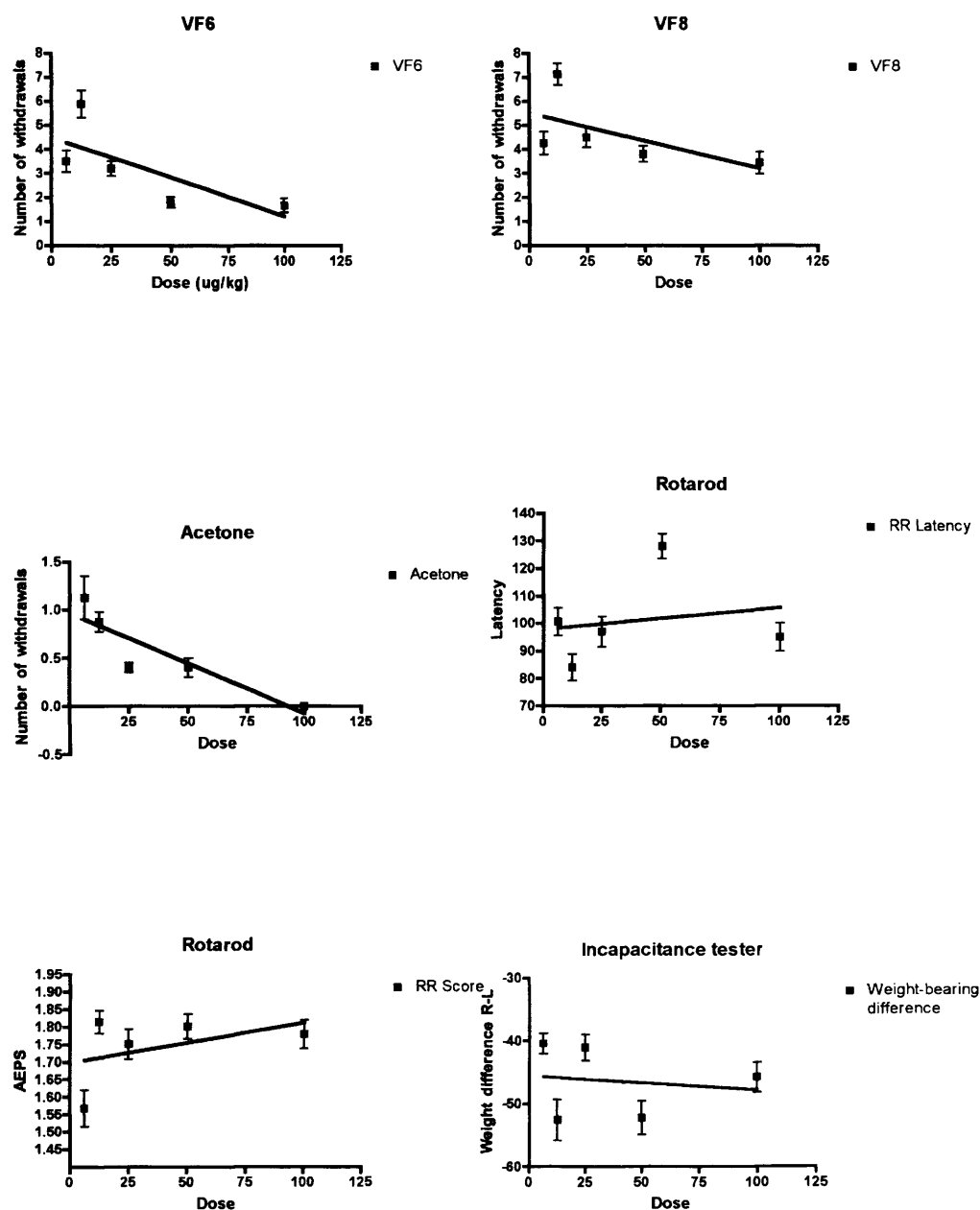


Figure 7.5. Determination of A006 ED50 concentrations, for each modality tested. Based on the maximum response per dose at any time-point.

## 7.3.3.2 Second method

The sponsor thought it would be more appropriate to avoid mixing the time-points or disregarding the baseline.

The percentage of inhibition in comparison to the baseline (pre-dosing) was thus calculated for each dose (6.25, 12.5, 25, 50 and 100 µg/kg) at each time point (0.5 h, 2 h and 4 h post-injection). Only results from the VF 6 g, VF 8 g and acetone tests could be used as they were the only ones for which there is some dose-response relationship.

Figure 7.6, Figure 7.7 and Figure 7.8 display the ED50 found for VF6, VF8 and Acetone respectively for each of the time point post-dosing.

ED50 (µg/kg)					
Test	14+0.5h	14+2h	14+4h	Average	SD
VF6	50	50	53.5	51.17	2.02
VF8	50	51.7	50	50.57	0.98
Acetone	52.2	50	53.8	52	1.91
				51.24	1.64
Average	50.73	50.57	52.43	51.24	
SD	1.27	0.98	2.11	1.45	

Table 7.3. ED50 averages.

For each time-point, very similar results were obtained, as indicated in the table above. Therefore all the values from all tests at all time-points were averaged and an ED50 of  $51.24 \pm 1.6$  µg/kg was found, which was not very different from the result found using the previous method,  $50.5 \pm 2.7$  µg/kg.

We have to note that the calculations 4 hours after AS006 injection might not be as accurate as for the other time-points for the VF testing (mechanical sensitivity) due to the weak dose-effect relationship at that time (cf. weak slopes). On the other hand, acetone responses still showed a strong dose-effect relationship even at 4 hours post-treatment.

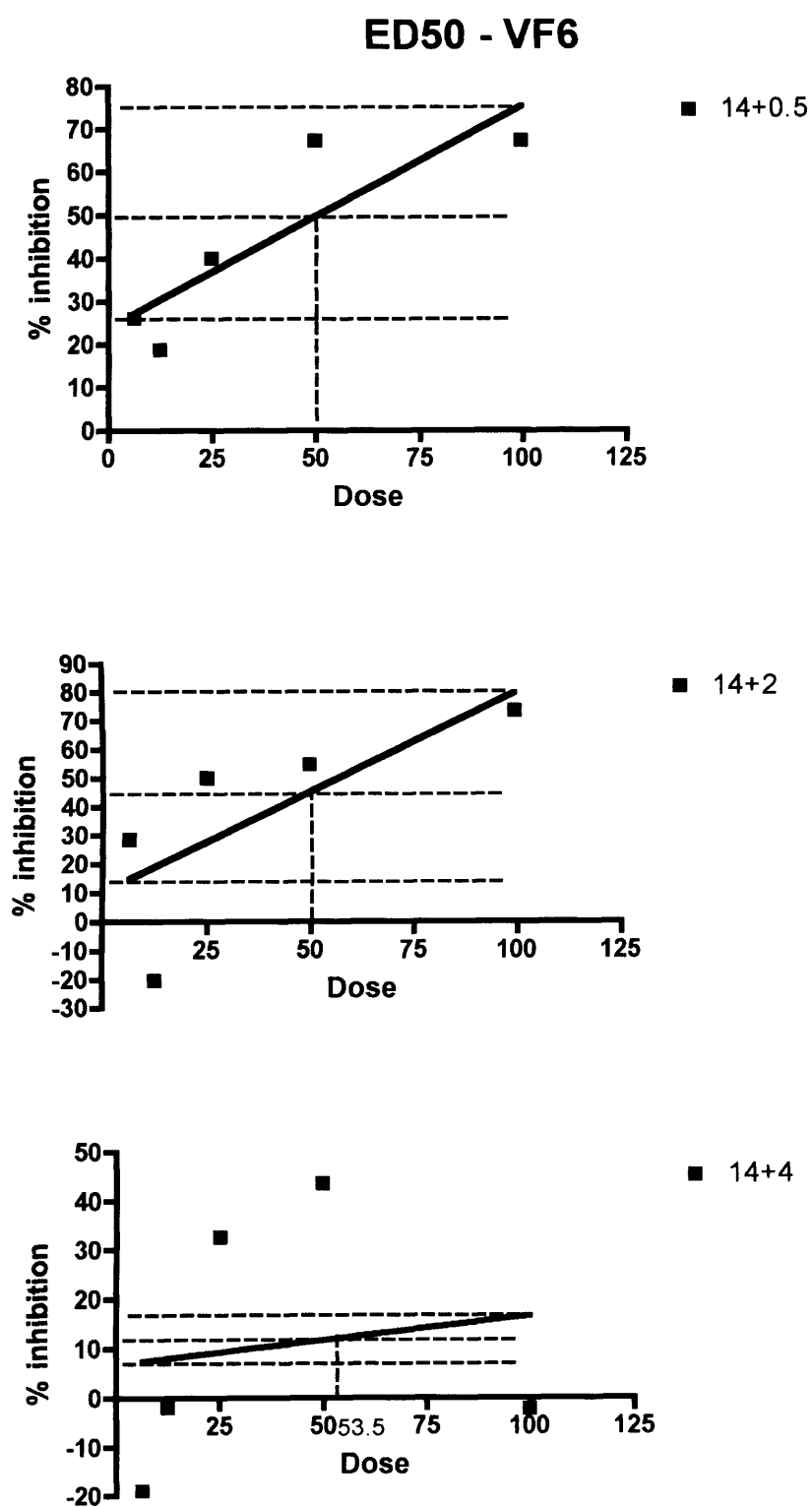


Figure 7.6. Calculation of ED50 for each time point using percentage inhibition of VF6 responses.

## ED50 - VF8

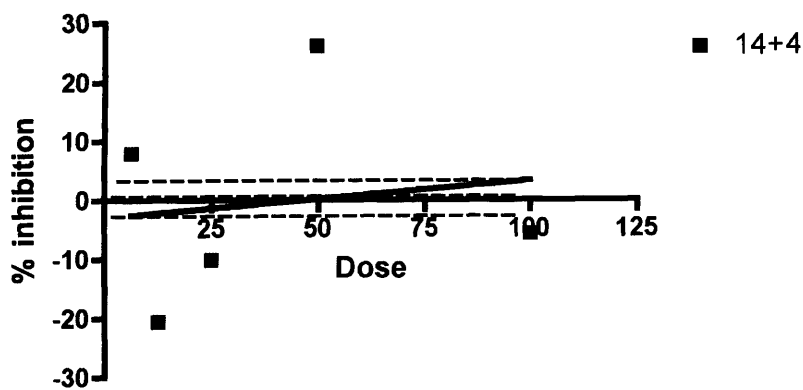
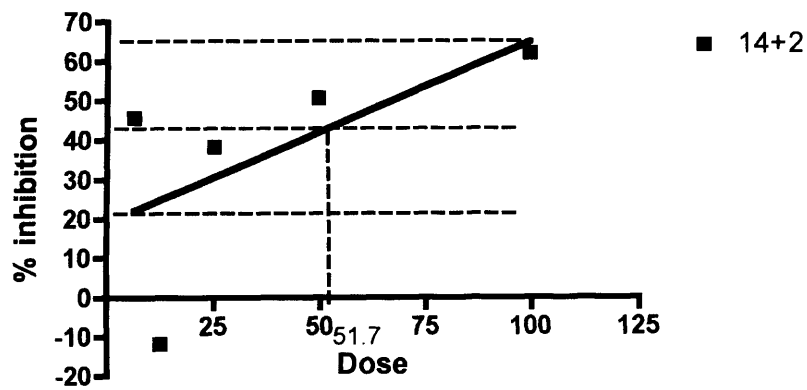
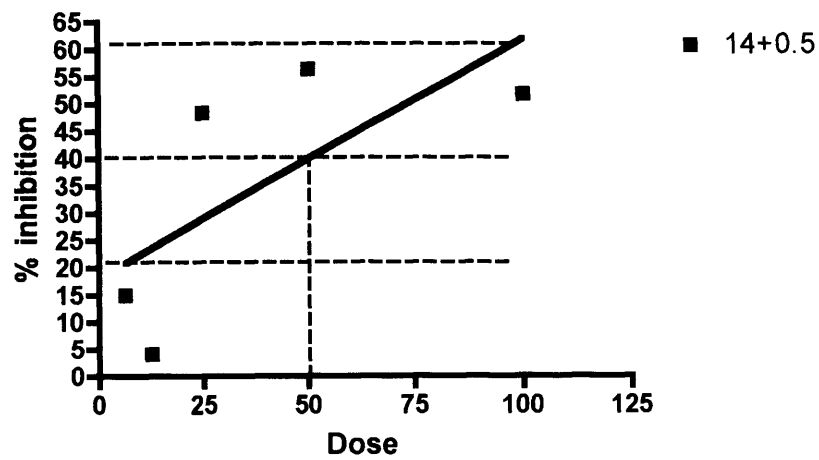


Figure 7.7. Calculation of ED50 for each time point using percentage inhibition of VF6 responses.



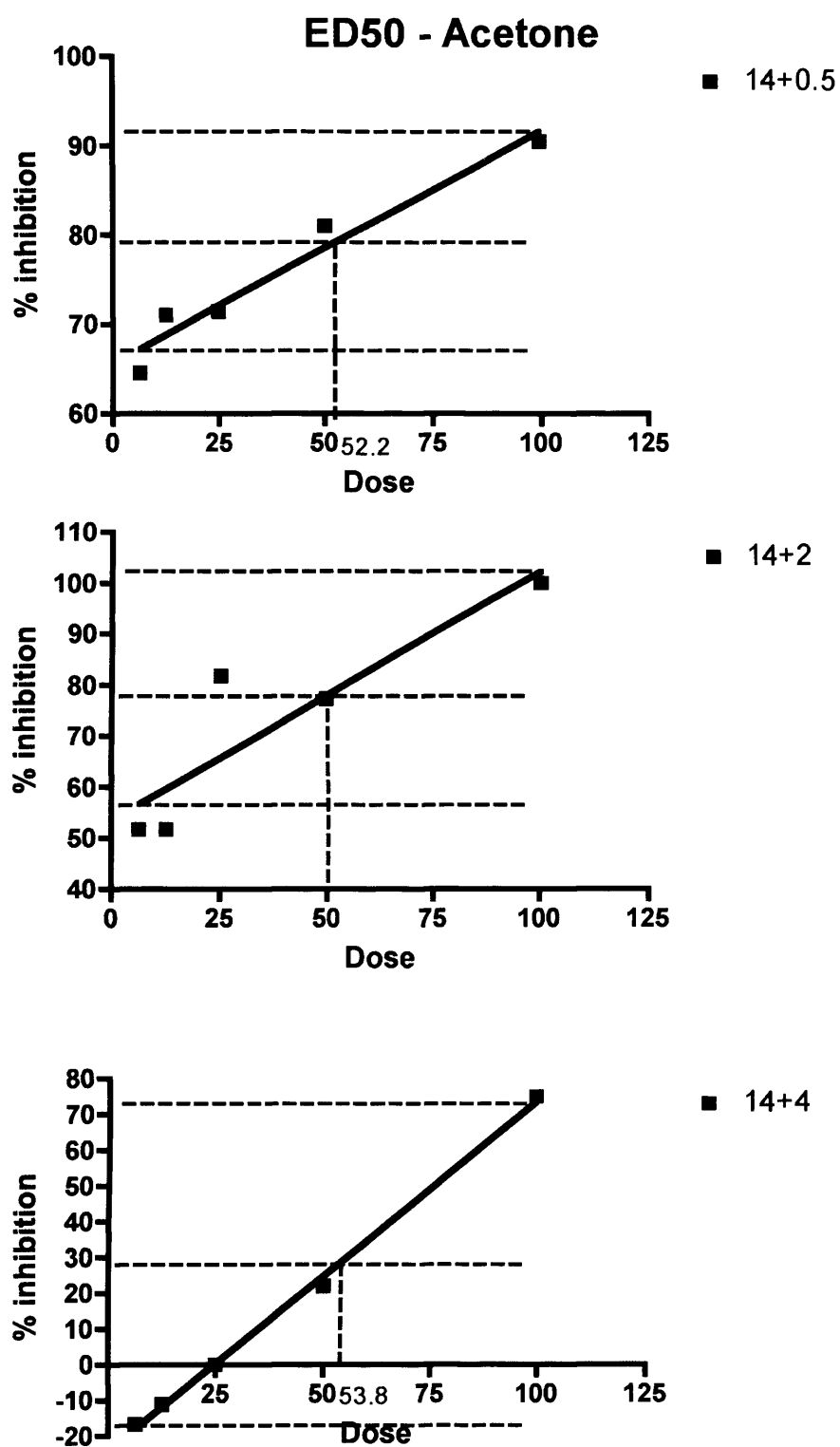


Figure 7.8. Calculation of ED50 for each time point using percentage inhibition of Acetone responses.

## 7.4 Discussion

AS006 had analgesic actions over a defined dose range and was positive in all modalities of tests used.

### 7.4.1 *Effects produced at different doses*

The compound AS006 had a clear and marked effect at reducing pain related behaviour (mechanical and cooling hypersensitivity, weight-bearing and ambulatory-evoked pain) for doses comprised between 25 and 100  $\mu\text{g/kg}$  for up to 2 hours post-injection with extended relief up to 240 minutes on some parameters.

The rotarod apparatus showed an increased latency to fall for the rats treated with 25-100  $\mu\text{g/kg}$  (but not with morphine) for up to 4 hours suggesting an improvement in their ability to walk on the rod with no sedation effect, further supporting the potential analgesic effects of the drug.

The use of lower doses of 12.5 and 6.25  $\mu\text{g/kg}$  showed a significant effect on the acetone responses at 30' post-injection, which tended to persist to 120 minutes. Inconsistent effects on mechanical sensitivity and weight-bearing were noted but no effect on ambulatory-evoked pain could be demonstrated.

It is important to note that for all the doses of AS006 used, immediately after injection, some animals appeared very still and calm. However this effect did not last very long: moving and tapping the cages would be enough to wake them up, and was over before randomly allocating the rats to the testing cages. Being so close in time to the injection, it is difficult to interpret the significance of this observation in terms of drug effect. In this context, it should be noted that the rotarod latency to fall at 30' post-injection was not affected except at the highest dose of 300  $\mu\text{g/kg}$ .

### 7.4.2 *Mechanism of action of AS006*

There is a clear therapeutic window in this model but it will be important to study all the other side-effects associated with the chronic and long-term use of opioids.

Because of the more peripheral rather than central action of the AS006 compound, some side-effects associated with the CNS such as tolerance or addiction may be avoided. However, other side-effects related for example to the gastro-intestinal system, in particular constipation, will need to be addressed thoroughly, as it is one of the first source of complaint (and termination of treatment) from patients treated with common opioids.

Centrally penetrating opioids such as morphine are known to exert their actions not only through central opioid receptors but also to play a substantial part on nociception through peripheral opioid receptors (D. Labuz et al., 2007).

In the periphery, opioid receptors have been identified in small diameter nociceptive primary afferent neurones. Following their synthesis in the cell bodies of these neurones in DRG, these receptors are intra-axonally transported into neuronal processes. Under conditions of tissue injury such as inflammation, neuropathy or bone damage, the number of opioid receptors in peripheral terminals is increased at the site of injury, resulting in enhanced agonist activity (S. A. Mousa et al., 2001; C. Stein et al., 2001; C. Stein et al., 2003).

The rotarod score and weight-bearing results, supposedly influenced by primary hyperalgesia, are in full agreement with these mechanisms, as nociceptive behaviour was decreased for up to 4 hours with AS006, and for up to two hours, to the same extent, with morphine. However the effects AS006 on mechanical and cooling sensitivity in the hind paw, area of secondary hyperalgesia, were of the same amplitude as those of morphine, for similar durations (up to 120 min).

In order to try and understand the mechanisms of action of AS006, it is useful to study the underlying mechanisms of primary and secondary hyperalgesia presented on Figure 7.9. Peripheral sensitisation of nociceptive fibres causes primary hyperalgesia, which in turn induces secondary hyperalgesia through central sensitisation. As a result, some A fibres, mostly low threshold mechanoreceptors but also some A fibre – but not C fibre – nociceptors, innervating an area outside the zone of injury also start to participate in the secondary mechanical hyperalgesia (E. A. Ziegler et al., 1999). Since A-delta and C fibres possess opioid receptors, peripheral actions of opioids on these fibre types would explain the observed behavioural effects.

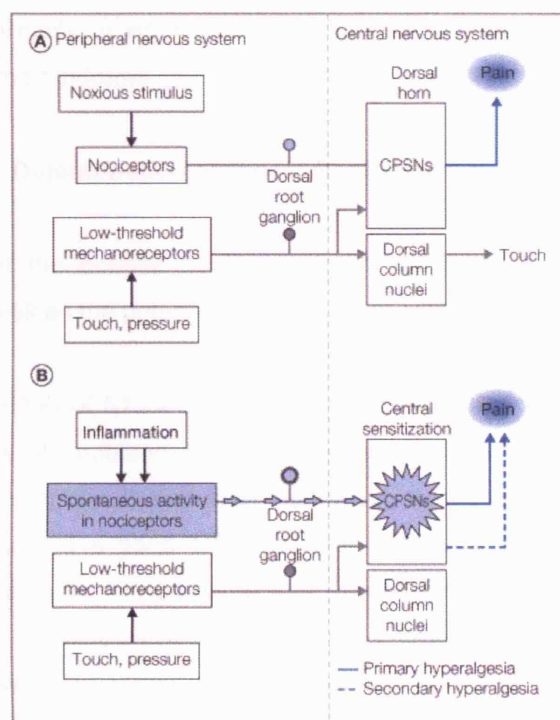


Figure 7.9. Development of secondary hyperalgesia through central sensitisation. A Nociceptors signal acute pain. Noxious stimuli selectively activate nociceptors that project to central pain-signalling neurons (CPSNs) in the spinal cord. The CPSNs project to higher centres where pain is perceived. Low-threshold mechanoreceptors convey the sensation of touch. B Injury or inflammation leads to sensitisation of primary afferent nociceptors. The enhanced responsiveness or sensitisation of primary afferents accounts for primary hyperalgesia. The nociceptors also develop spontaneous activity, which drives the development of sensitisation of the CPSNs. This central sensitisation involves an enhanced connectivity between low-threshold mechanoreceptors and CPSNs. Now, signals from low-threshold mechanoreceptors gain access to the pain pathway, leading to the development of secondary hyperalgesia to mechanical stimuli (From McMahon & Koltzenburg: Wall and Melzack's Textbook of Pain 5e – [www.textbookofpain.com](http://www.textbookofpain.com)).

AS006 at the doses used in this study is likely to be restricted to the peripheral nervous system as it does not notably cross the blood brain barrier. The upregulation of mu opioid receptors, on which it acts and that can produce analgesic effects, is well known in small diameter nociceptive fibre, mostly C and A-delta innervating the injured area.

AS006 can thus act directly on those receptors and reduce primary hyperalgesia, which was verified by the incapitance tester and the rotarod pain score.

AS006 also reduced pain in the paw, area of secondary hyperalgesia. The first hypothesis could be that it is mediated by a direct action of AS006 on some A fibre nociceptors. Another hypothesis could be that the actions of AS006 on C-fibres innervating the damaged zone of primary hyperalgesia would at the same time decrease the central sensitisation of second order spinal projection neurones: secondary hyperalgesia could

thus be decreased. Finally, it would be very likely for both mechanisms to occur and produce analgesia in the hindpaws.

#### **7.4.3 Determination of the ED50 of AS006 in this model**

The determination of the optimal dosing regimen will have to take the mechanisms described above, as well as the potential side-effects at higher doses, into account.

Finally, an average ED50 of  $51.24 \pm 1.6 \mu\text{g/kg}$  was calculated for AS006 in this model, using the percentage of inhibition at all time-points and using VF6, VF8 and acetone results. No ED50 for morphine is known in this model, as morphine has already been well characterised and showed a good effect without any sedation effect at the dose of 3 mg/kg. However, it was interesting to note that, whereas the effects of morphine seemed to be maximal around 30 minutes post-injection, the effects of AS006 (at the higher doses, Part A of the study) were extended up to 120 minutes post-injection for the acetone and VF8 responses (Figure 7.1), and even up to 240 minutes post injection for the rotarod latency (Figure 7.2.A).

#### **7.4.4 Usefulness of the model to identify new pharmacological agents**

Recently, the MIA-model of OA was used to assess the effects of lacosamide, a member of a family of functionalised amino acids that are analogues of endogenous amino acids and D-serine and to compare it with morphine and diclofenac. Lacosamide was shown to reduce pain behaviour at 3, 7 and 14 days post-OA induction (B. Beyreuther et al., 2007).

These studies confirm the usefulness of this model at assessing the pharmacological effects of new analgesic compounds.

## **Chapter 8. General discussion**

In summary, the experiments performed have validated the MIA-induced model of OA pain as a reproducible and shown it to be a useful preclinical tool to test new pharmacological agents. The investigation of changes in mechanical and cooling hypersensitivity, as well as ambulatory evoked pain score and latency to fall and hindpaw weight-bearing distribution can provide complementary information about the animals' nociceptive state.

This is illustrated by the fact that both morphine and gabapentin were shown to be efficacious in this model of OA pain, and additionally the differences in their pharmacological profile were outlined.

## **8.1 Development of primary and secondary hyperalgesia in this model**

### **8.1.1 *Underlying mechanisms***

The development of both primary and secondary hyperalgesia-like mechanisms have been studied in various experiments using a battery of different tests. In order to explain and discuss my results as a whole, it is important to keep the possible underlying mechanisms in mind. Peripheral sensitisation of nociceptive primary afferents in the zone of injury results in primary - mechanical and thermal - hyperalgesia, which can then result in an expanded receptive field corresponding to an area of secondary – mechanical – hyperalgesia whose mechanism is presented on Figure 8.1.

Briefly, the development of secondary hyperalgesia relies on the induction of a heterosynaptic facilitation by C-fibre nociceptors (including polymodal C-fibre nociceptors, mechanically insensitive afferents, chemoheat nociceptors and chemospecific nociceptors) innervating the zone of primary hyperalgesia (for example the knee) of mechanosensitive nociceptive transmission from A fibres – both low threshold mechanoreceptors (responsible for secondary hyperalgesia for light touch and nociceptors (responsible for secondary hyperalgesia for punctate stimuli) – from the area of secondary hyperalgesia (the hind paw) to second order projecting neurones into the spinal cord. C-fibre input in the area of secondary hyperalgesia is not known to be facilitated.

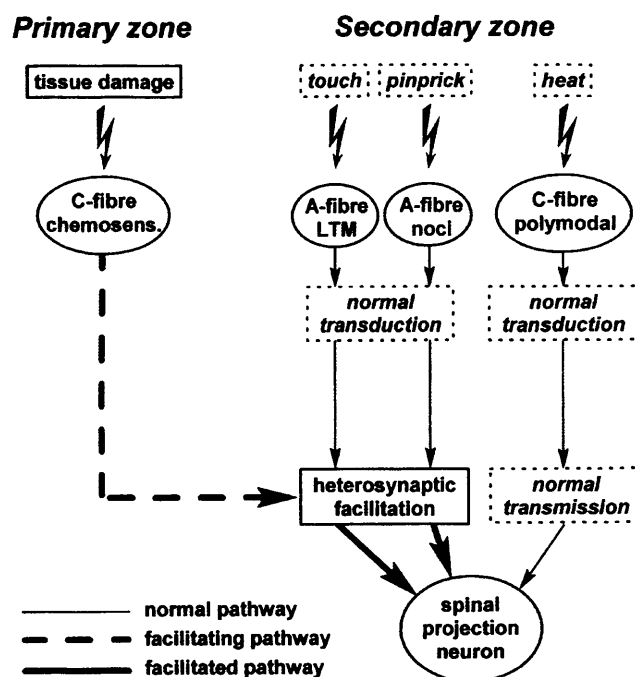


Figure 8.1. Model for cutaneous secondary hyperalgesia in humans. The facilitating pathway involves capsaicin-sensitive C-fibre nociceptors in injured tissue. These afferents are known to discharge vigorously to capsaicin injection and to induce sensitisation of nociceptive neurons in the dorsal horn of the spinal cord. This sensitisation is heterosynaptic and leads to facilitation of two other inputs onto spinal cord nociceptive projection neurones. The first facilitated pathway involves low-threshold mechanoreceptors (A-fibre LTM) that normally signal touch sensation. The second facilitated pathway involves A-fibre nociceptors, including high-threshold mechanoreceptors (A-fibre HTM) and A-fibre nociceptors with high heat thresholds (type I AMHs). The absence of secondary hyperalgesia under established A-fibre conduction blockade suggests that C-fibre inputs themselves are not facilitated. The absence of heat hyperalgesia in the secondary zone suggests that inputs from heat-sensitive nociceptors in general are not facilitated, including polymodal A- and C-fibre nociceptors and A-fibres that normally signal first pain to heat (type II AMHs). (From E. A. Ziegler et al., 1999).

### 8.1.2 Characterisation of primary hyperalgesia

The development of primary hyperalgesia was successfully assessed using the rotarod, with the ambulatory pain score, as well as with the incapacitance tester investigating weight-bearing changes, but only for the AS006 study.

In those studies, primary hyperalgesia developed gradually and smoothly, with no apparent biphasicity.

We have to bear in mind that it was an indirect measure of primary hyperalgesia, as tests directly applied onto the knee joint were not performed. Indeed, we have to assume that the incapacitance tester and the rotarod tests are correlated with pain from the knee joint and can give us a fair idea of the changes in nociceptive behaviour.



Unfortunately, primary hyperalgesia could not be recorded using electrophysiological methods due to material and time limitation. However, it will be interesting to study those changes.

### 8.1.3 Characterisation of secondary hyperalgesia

The assessment of secondary hyperalgesia was performed both using behavioural tests and using *in vivo* electrophysiology. It seemed to develop in a biphasic manner and the acetone and von Frey hairs results had more variation than the incapacitance or rotarod ones. Here I will try to explain what we might be recording in the hindpaw, as it is likely that secondary hyperalgesia is not the only explanation.

Because pain has both a sensory and an affective component, it is difficult to know what the tests carried out were actually measuring in live animals.

Indeed, in chronic pain states, there is often a discrepancy between what patients can expect (healing, reduction of pain) and what actually happens (lasting or increasing pain), which can lead to catastrophising and pain-related fear. A cognitive-behavioural model of such pain related fear is presented on Figure 8.2.

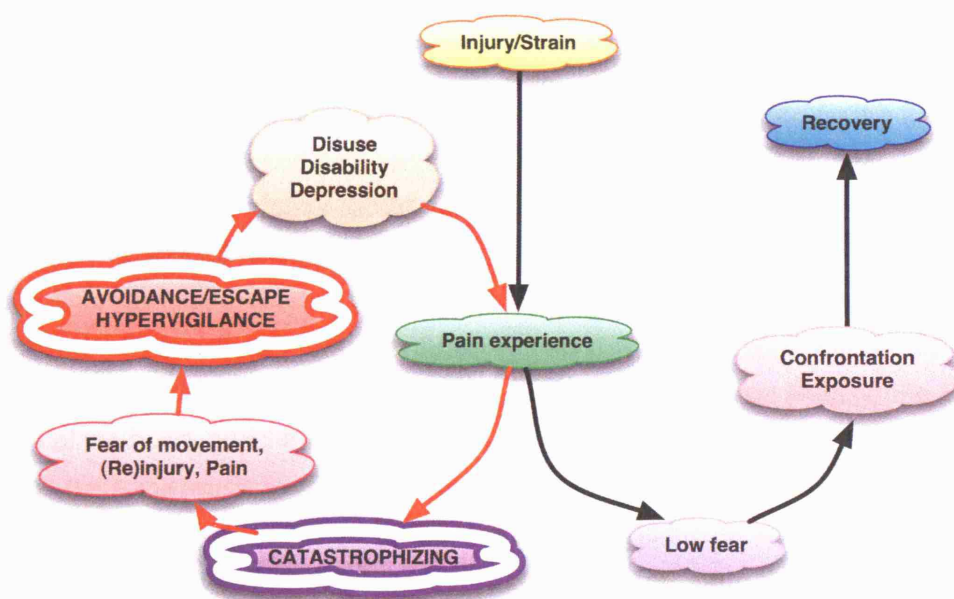


Figure 8.2. Cognitive-behavioural model of pain-related fear (Adapted from J. W. Vlaeyen et al., 1995).

Pain related fear usually begins when pain, either caused by an injury or strain, is perceived as a threat, which can lead to pain catastrophising. This is due to the fear of movement, (re)injury or simply because of the pain itself, various subsequent responses can ensue, including avoidance and escape, or hypervigilance, followed by disability, disuse of the painful limb, depression and persistent chronic pain experiences. This leads to the instalment of a vicious circle of increasing fear and avoidance. In non-catastrophising patients, or in situations where healing rapidly occurs following an acute pain state, pain-related fear is unlikely and normal activities can be resumed rapidly, following a smooth recovery period (J. W. Vlaeyen et al., 1995).

The natural question following the presentation of this model of pain-related fear is to ask whether this happens in animal models of pain as well.

In the MIA-induced model of OA, the behavioural responses were recorded following stimulation of the hindpaw, in particular for the von Frey hairs and acetone drop testing, as well as for the *in vivo* electrophysiology. What was actually being measured in those situations, or what provoked the responses?

Those questions have been discussed partly in Chapter 3 regarding the model validation and its pharmacological modulation using morphine and gabapentin, but additionally here I will hypothesise and endeavour to give some explanation in respect to what was obtained with hindpaw stimulation.

The first hypothesis is that because of central sensitisation incurred by the knee joint OA chronic pain state, referred pain can be measured from the foot. Indeed, hyperalgesia from deep and visceral structures can be referred to somatic structures. The textbook example of that is myocardial infarction, or commonly referred to as heart attack, which follows the interruption of blood vessels of the heart. The classical symptoms of acute myocardial infarction are chest pain that is typically radiating to the left arm, shortness of breath, nausea, vomiting, palpitations, sweating, and anxiety. We can thus imagine the knee in the place of the heart, and the foot in the place of the arm, and the resulting referred pain. The neural mechanisms underlying referred pain have been investigated and different theories exist. As shown below, these include the – now discounted - axon reflex theory that assumes convergence of somatic and visceral primary afferents before they enter the spinal cord (D. C. Sinclair et al., 1948), the spinal convergence-projection theory, the generally accepted explanation of referred pain, which assumes convergence of somatic and visceral primary afferent neurones onto common dorsal horn second-order neurones (T. C. Ruch, 1946), the spinal convergence facilitation theory, explaining referred pain by visceral afferent fibres capable of facilitating the projection of activity in somatic afferents

to the brain (J. MacKenzie, 1909) or supraspinal interaction theory suggesting a somato-visceral interaction at a cerebral level (G. W. Theobald, 1941).

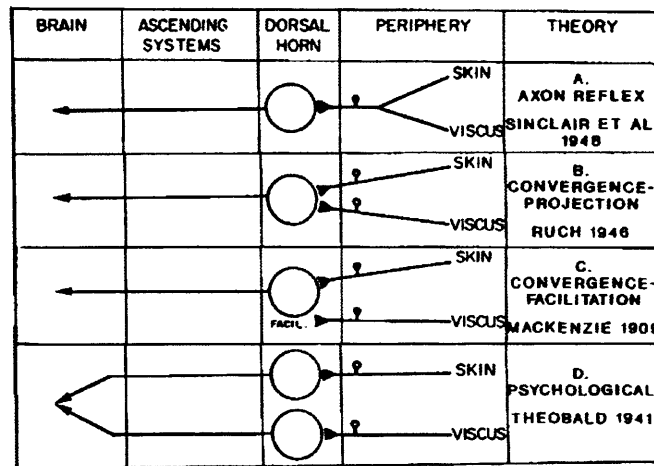


Figure 8.3. Theoretical mechanisms underlying referred pain (From J. F. B. Morrison, 1987).

The second simple hypothesis for responses recorded in response to hindpaw stimulation following OA induction in the knee is that by stimulating and mobilising the hindpaw (during behavioural or electrophysiological testing), pain was evoked through movement transmitted to the aforementioned inflamed or injured knee.

The third hypothesis relies on the cognitive-behavioural model of pain-related fear explained above (Figure 8.2) and applied to rats, where responses in response to stimulation of the hindpaw could be provoked through a general state of increased attention or hypervigilance.

Finally, the fourth controversial hypothesis would be peripheral sensitisation of the areas of primary hyperalgesia expanding to the area of secondary hyperalgesia with little central component. Indeed plasticity in primary afferents could give some mechanoreceptors access to the nociceptive neurones by means of a presynaptic link (F. Cervero et al., 2003).

#### 8.1.4 Pharmacological modulation

The different tests used to assess behavioural changes were very informative regarding the effects of the different drugs.

Again there seemed to be more clean-cut less variable results with the tests mainly investigating primary hyperalgesia than with the ones studying secondary hyperalgesia.

However, the latter still worked very well and provided useful information in terms of mechanical and cooling hypersensitivity for example. It is important to bear in mind that those tests can only help us to have an idea of what might be happening and that they are complementary to each other, helping us to unravel this complicated jigsaw.

Even tests such as the latency to fall recorded using the rotarod, which seemed to be devoid of useful information in non-pharmacological studies, later proved to be important when studying sedation or locomotor impairment due to a gabapentin for example.

## 8.2 Nature of the pain in the MIA-induced model

The studies carried out also investigated the role of inflammation in the model and showed that it was most likely to be involved during the first week after the MIA injection. The NSAID meloxicam demonstrated a variable efficacy on the chronic pain state from day 14 onwards, which would be in agreement with the fact that inflammation was not a constant but a more sporadic and earlier characteristic of OA.

The investigation of ATF-3 immunoreactivity coupled with Fast Blue retrograde labelling did not reveal any significant neuropathic damage involved following MIA injection.

These findings could be summarised by placing the MIA-induced model of OA on the diagram below.

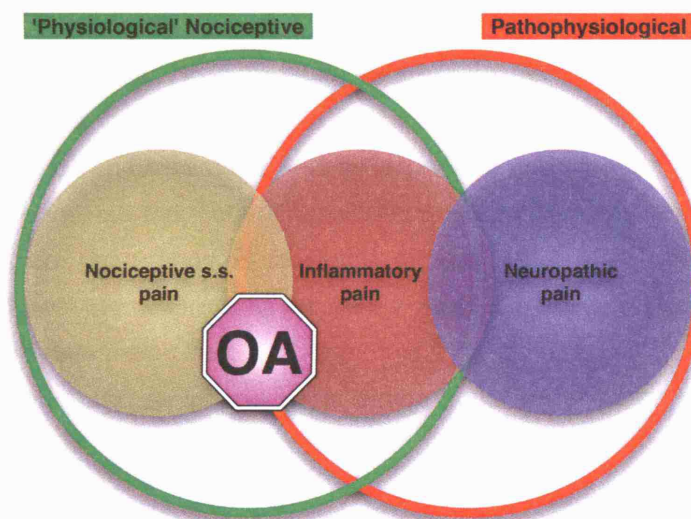


Figure 8.4. Nature of the pain in our model of OA among different types of pain (Adapted from McMahon & Koltzenburg: Wall and Melzack's Textbook of pain 5e – [www.textbookofpain.com](http://www.textbookofpain.com)).

Indeed, after being called “a model of chronic nociceptive pain” (R. Combe et al., 2004) and with intermittent inflammatory peaks, it is likely that the pathophysiological chronic pain state studied with this model is a result of the combination of both nociceptive and inflammatory pain, which would be in agreement with what is seen in osteoarthritis patients, where inflammation is not present all the time contrary to rheumatoid arthritis patients.

### 8.3      **Neuronal mechanisms in this model of OA pain**

Following the identification of the different types of pain involved in this model, *in vivo* electrophysiological studies provided some information about changes in neuronal responses.

#### 8.3.1   ***A minor role for central sensitisation?***

Although behavioural studies demonstrated clear differences between arthritic and sham rats, spinal cord deep wide-dynamic range neuronal responses were not shown to be statistically different between those two groups, albeit the existence of a trend in that direction for mechanical responses was seen. Again, these results – albeit not being significant – are in agreement with the development of a secondary hyperalgesia-like phenomenon.

The ablation of superficial NK-1 receptor expressing neurones did not produce any statistically significant change, neither in terms of behavioural nor neuronal responses, although there was a trend towards decreased responses.

These findings suggested that central sensitisation played a less important role when the electrophysiological recordings were made on day 14 post-OA induction and that superficial NK-1 receptor expressing neurones might play a less important role in this model of OA and inflammation than in others.

The peripheral drive and peripheral sensitisation could be of major importance in this chronic OA pain model.

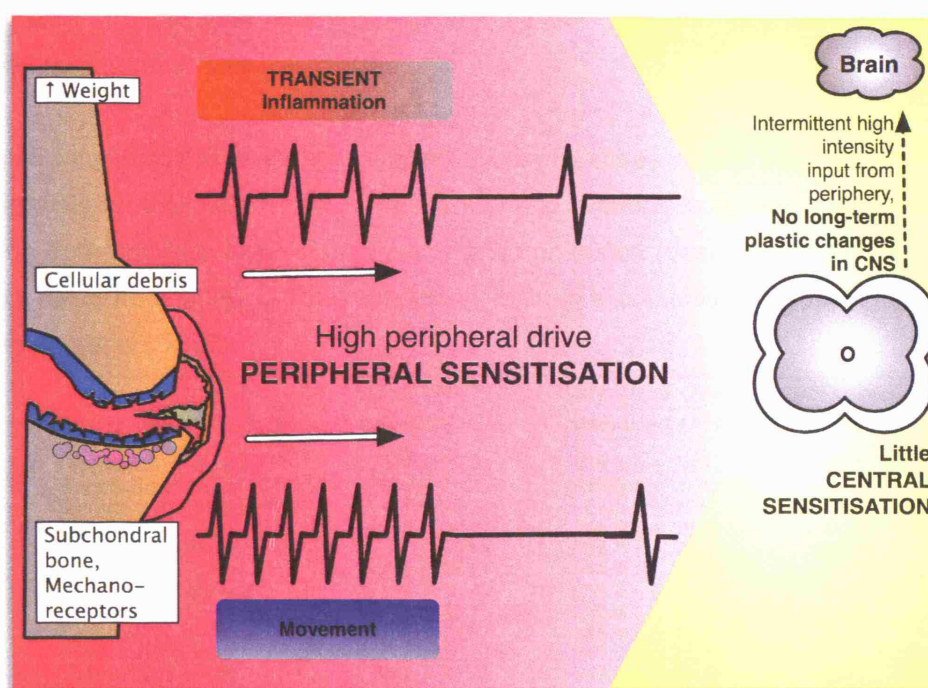


Figure 8.5. The relative roles of peripheral and central sensitisation in the MIA-induced model of OA. The high peripheral drive, increased by sporadic inflammatory events or evoked by movement, seems to be more important than central sensitisation in this chronic pain condition, with minimal CNS plastic changes.

Indeed, in the MIA model pain could be elicited by temporary peaks of inflammation. In the meantime, movement-evoked pain, the other major source of pain, could be kept at a minimal level, through avoidance of usage of the lesioned limb by the animals. Inflammation and movement taken together could then underlie the peripheral sensitisation state. However, the intensity of such stimulation would never been high enough to induce changes in the spinal cord and *a fortiori* in the brain.

Indeed, contrary to other pain conditions such as phantom limb pain or other refractory neuropathic pain states where pain signals from the periphery seem to have “burnt their way” into the central nervous system in a permanent manner, as pain does not cease with the disappearance of the peripheral input. OA pain, like other painful conditions such as an entrapped nerve, the passage of a kidney stone or childbirth tends to stop rapidly once the peripheral source of pain is removed, i.e. following joint arthroplasty/replacement surgery, release of the entrapped nerve, removal of the stone or childbirth itself respectively, with no obvious permanent trace. In those states, central sensitisation does not appear to be induced or to be irreversible. In OA, knee or hip arthroplasty usually involves some pain,



but as post-surgical pain resolves, the arthritic pain will normally have disappeared, provided no iatrogenic pain was caused.

It is also interesting to study the causes of pain in knees of OA patients. Despite the heterogeneity of the pain symptoms, early morning stiffness is common, as well as pain resulting from activities involving knee extension or flexion (described in the table below) such as rising from sitting, walking, shopping, or menial tasks such as putting clothing on (P. Creamer et al., 1998).

	Medial (N=23)	Generalised (N=35)	P value
Heavy domestic duties	Medial (N=23)	Generalised (N=35)	P value
Descending stairs	52.7 (± 29.7)	56.5 (± 26.7)	0.62
Ascending stairs	47.7 (± 29.7)	53.5 (± 27.6)	0.45
Rising from sitting	38.6 (± 29.8)	57.6 (± 25.9)	0.01
Standing	36.7 (± 29.1)	46.9 (± 28.6)	0.20
Bending to floor	32.3 (± 29.4)	58.8 (± 27.3)	0.0009
Walking	29.7 (± 22.7)	41.9 (± 26.2)	0.07
Getting in/out car	37.0 (± 28.4)	55.4 (± 22.0)	0.007
Shopping	40.0 (± 28.5)	57.6 (± 31.0)	0.03
Putting on socks	28.0 (± 24.9)	48.4 (± 31.3)	0.01
Rising from bed	31.3 (± 29.2)	47.7 (± 26.7)	0.03
Taking off socks	27.6 (± 24.2)	47.4 (± 29.5)	0.01
Lying in bed	23.3 (± 26.2)	32.1 (± 26.0)	0.22
Getting in/out bath	34.2 (± 38.0)	53.3 (± 30.9)	0.04
Sitting	22.9 (± 24.2)	32.0 (± 23.1)	0.16
Getting on/off toilet	21.4 (± 23.7)	40.9 (± 27.7)	0.008
Heavy domestic duties	49.0 (± 29.1)	64.3 (± 28.1)	0.05
Light domestic duties	29.1 (± 26.9)	37.0 (± 24.8)	0.26

Scores may range from 0–100 with higher scores indicating greater disability. *P* values given for differences between groups (*t*-test).

Table 8.1. WOMAC function scores (unadjusted mean ± S.D.) for participants with 'medial' and 'generalised' knee pain (From P. Creamer et al., 1998).

The peripheral drive to generate pain was shown to be present in approximately 60–80% of patients depending on the affected joint by intra-articular studies using a local anaesthetic and by studying pain resolution following total joint arthroplasty in hip and knee OA (P. Creamer et al., 1996; R. W. Crawford et al., 1998; O. Ethgen et al., 2004). Following knee and hip arthroplasty, comorbidities such as anxiety and depression were highlighted in patients with modest post-surgical improvement (O. Ethgen et al., 2004). These results suggest that even in humans, the peripheral drive is the main actor in this chronic pain state, more than central sensitisation.

As a result, it is important to highlight some known key factors of peripheral and central sensitisation and how they could be pharmacologically modulated to improve pain treatment.



### 8.3.2 *Peripheral mechanisms*

Various receptors expressed at the surface of primary sensory neurones can contribute to pain and to sensitisation. In my studies on this model of OA pain for example, in order to decrease pain transmission, EP2 receptors and VGCCs activation has been indirectly antagonised through the limitation of PGE2 production and the administration of NSAIDs/corticosteroids and gabapentin respectively, whilst mu-opioid receptors (MOR) could become activated by morphine. Other receptors such as TRPV1, the “chilli receptor” activated by heat, capsaicin and protons, are sometimes targeted with the topical application of capsaicin gels, aiming at TRPV1 receptor desensitisation and decreased pain. OA patients also like to keep their OA joint warm, which might not activate those receptors.

Other receptors are also known to be expressed on primary sensory neurones: the bradykinin receptor 2, P2Y and P2X receptors of ATP, proton-activated ASIC channels, or TRPA1 and TRPM8 recently shown to be activated by formalin and both menthol and icilin respectively (A. Dhaka et al., 2007; C. R. McNamara et al., 2007). Their role in OA pain is not well characterised although *in vivo* electrophysiological studies in the carrageenan model of inflammation suggested a role for spinal P2X receptors in the modulation of spinal nociceptive transmission (L. C. Stanfa et al., 2000).

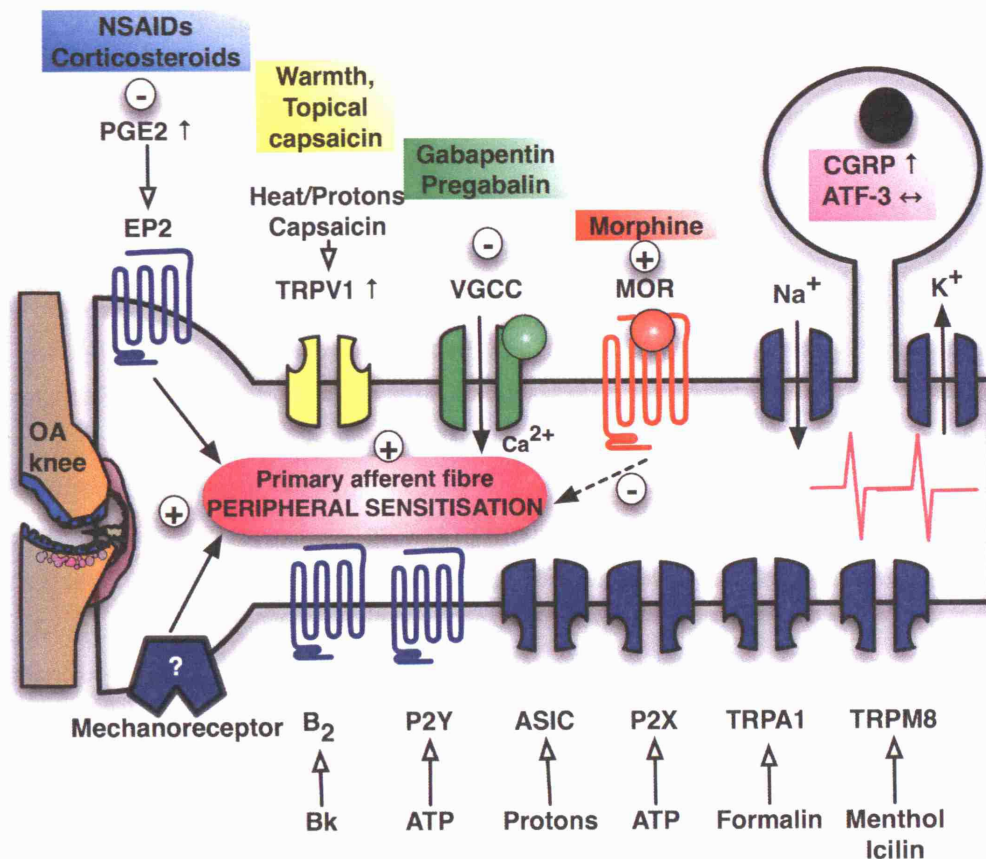


Figure 8.6. Nociceptors, receptors and cellular events potentially involved in peripheral sensitisation of primary afferent fibres in the OA condition, and their pharmacological modulation.

Little is known about pain mechanoreceptors and how mechanical noxious stimuli are encoded.

Na<sup>+</sup> and K<sup>+</sup> channels are mainly involved in the action potential generation and propagation. The NaV 1.8 channel, because of its highly restricted expression pattern to nociceptive peripheral sensory neurones could constitute a very interesting target to attenuate pain (A. N. Akopian et al., 1996; L. Djouhri et al., 2003).

The lack of ATF-3 up-regulation coupled with a reported increased expression of CGRP (J. Fernihough et al., 2005) in the spinal L4 DRGs has also been added to this diagram.

### 8.3.3 *Central mechanisms*

Mechanisms underlying activation of secondary order projecting neurones and central sensitisation are put into the OA pain model context in Figure 8.7.

When primary sensory neurones are activated in the periphery, various excitatory neurotransmitters such as glutamate or substance P are released into the synaptic cleft. In addition, in some pain states, serotonin release from descending pathways originating in the brainstem can also activate pre-synaptic 5HT-3 receptors and enhance the pre-synaptic cell excitability.

In response to glutamate release, NMDA, AMPA and metabotropic glutamate post-synaptic receptors are activated. Likewise, SP release can activate the NK-1 receptor and PGE2 the EP2 receptor on the projecting neurone. These phenomena not only trigger depolarisation of the membrane and activation of voltage-gated calcium channels, but they can also contribute to central sensitisation by downstream mechanisms already presented in the introduction, via transcription factors for example. Alternatively, the release of GABA by some inhibitory interneurons can activate GABA receptors and participate in the hyperpolarisation of the cell membrane, acting against cell activation. The production of PGE2 can also lead to the inhibition of the  $\alpha$ -3 subunit of glycine receptors, which can participate to the inhibition of glycinergic neurotransmission but also to pain sensitisation in response to inflammation (R. J. Harvey et al., 2004).

What can be done to modulate the activity of these receptors and thus central sensitisation? Morphine can bind to both pre- and post-synaptic MORs, which will result in an overall decreased neuronal excitability. Ondansetron, a selective 5HT-3 receptor antagonist, administered intrathecally was shown to mimic the effects of chemically ablating superficial NK-1 receptor expressing neurones and reduced neuronal excitability (R. Suzuki et al., 2002), although its effect might be negligible in this model of OA pain given the small changes seen following NK-1 receptor chemical ablation with SAP-SP. Gabapentin as well as pregabalin, by binding to the  $\alpha$ -2-delta-1 subunit of VGCCs, can also diminish neuronal hyperexcitability. Finally, NSAIDs and corticosteroids could inhibit the production of PGE2 in the CNS, thus preventing both the activation of EP2 receptors and the inhibition of glycinergic transmission via the  $\alpha$ -3 subunit of glycine receptors.

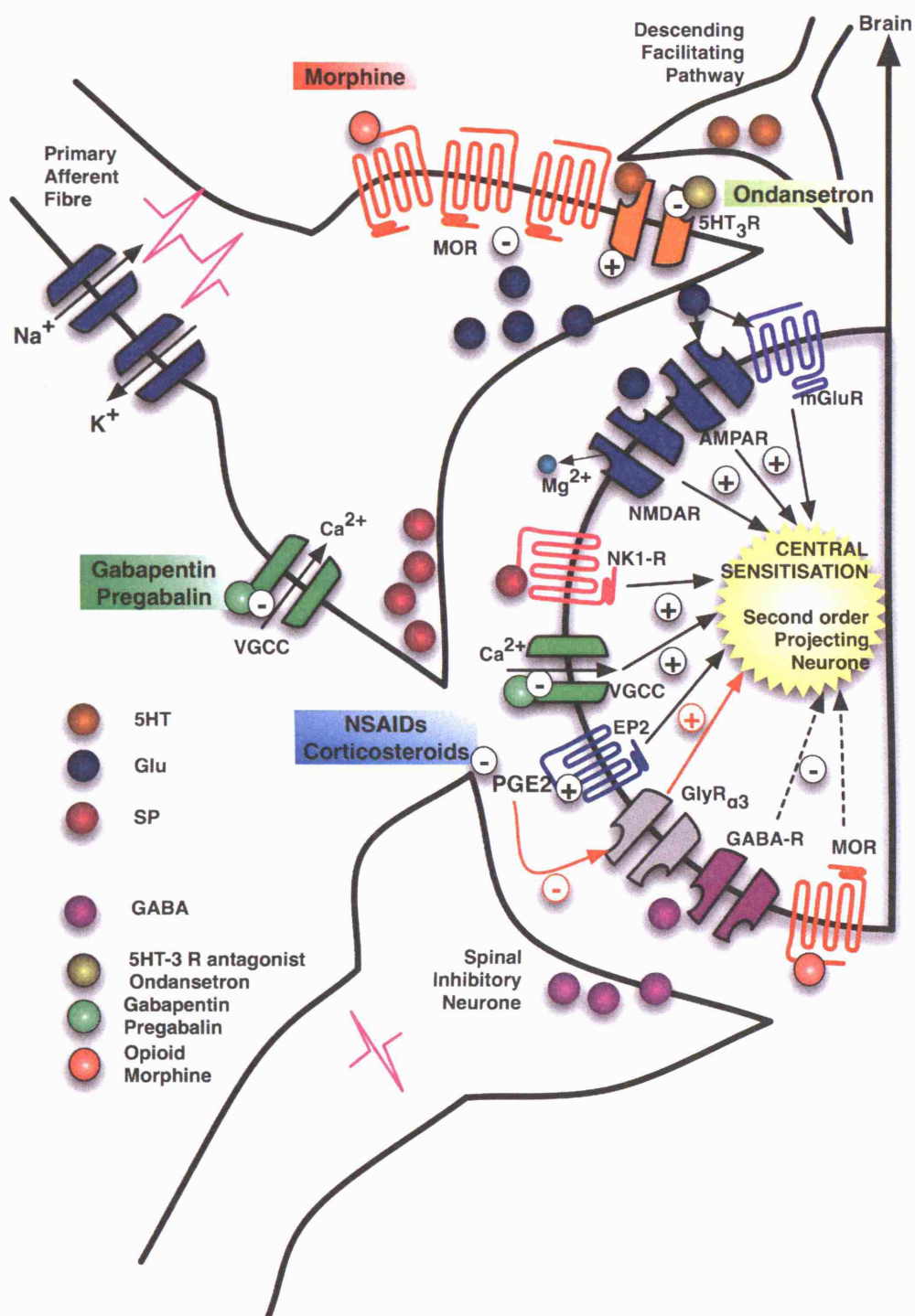


Figure 8.7. Neurotransmitters, receptors and events potentially involved in the activation of secondary projecting neurones and *in fine* to central sensitisation.

## 8.4 Current and future of OA treatment

### 8.4.1 *Existing treatment*

As presented in the introduction, OA treatment is currently purely symptomatic as no disease-modifying drugs are yet available.

Non-pharmacological measures such as weight loss can provide pain relief: 1 pound in weight reduction equates to a 4-pound reduction through joint, although it might not be easy to apply in all patients. The latter also use warm water as a mild pain homemade remedy. Exercise is also an important part of the treatment in order to limit muscle waste and increase the tonus and strength of muscles stabilising the joint.

Paracetamol and NSAIDs constitute the first line treatment of for OA patients, with their associated side-effects. Topical NSAIDs might be more effective than placebo (S. Biswal et al., 2006), as high concentrations can be reached inside the joint but they have to be applied everyday and patients can be non-compliant.

COX-2 selective inhibitors present fewer side-effects. However they have been under scrutiny following Vioxx™ withdrawal by Merck. More recently, in November 2007, the licence of Prexige™ (lumiracoxib) another COX-2 selective inhibitor medication currently in development by Novartis and used for OA treatment, was suspended by the Medicines and Healthcare products Regulatory Agency (MHRA) due to liver damage concerns.

The Commission on Human Medicines found an increase in the number of serious liver reactions among patients taking a 100-mg dose of the drug. Prexige™ (lumiracoxib) has been available in the UK since late 2005. Around 5,000 patients in the UK have been prescribed Prexige™, which helps ease the pain of osteoarthritis, particularly in the knee. It was already restricted for use in patients with liver problems. In Britain, there have been 23 reports of suspected adverse reactions since March 2006 - three of which were related to liver problems (Source: Novartis website).

Opioids remove the unpleasantness of pain but have serious side-effects such as constipation, nausea and cognitive impairment which can explain why some patients choose to discontinue their use of opiate analgesics and why many clinicians tend not to prescribe these drugs. An additional factor is fear of addiction and this relates to the potential use of peripherally acting opioids as described in Chapter 7.

No neuropathic pain component seemed to be involved in this model of OA pain. However, because of their central effects, and by increasing patients' quality of life (sleep for example), gabapentin and pregabalin could be useful drugs to test in humans. Moreover, it is not excluded that some nerve damage might occur at late OA stages in humans or following denervation in rats (P. T. Salo et al., 2002). Loss of knee afferents was also found in aging mice (P. T. Salo and W. G. Tatton, 1993). All these arguments could justify further the use of those drugs.

Intraarticular injections of steroids or hyaluronic acid can be sometimes useful but cannot be used as a routine treatment because of the iatrogenic risks (associated with the procedure itself).

Joint replacement is the ultimate therapy, although it is expensive and artificial joints are not designed to remain in place indefinitely and thus need to be replaced at regular time intervals, which limits their use, in particular in younger patients.

#### **8.4.2 *Future of pain and OA pain treatment***

Thus what is the future for pain therapy, in particular that of OA pain management? Will there be a single magic bullet for each chronic pain condition?

That is what some pharmaceutical companies were trying to do with the example of TRPV1 antagonists. TRPV1 is upregulated in some chronic pain models such as the MIA-induced OA pain model (J. Fernihough et al., 2005) but not in others like the antigen-induced arthritis model (K. J. Bar et al., 2004), and is normally downregulated in the uninjured animals. The release of various inflammatory mediators following tissue inflammation was shown to sensitise the TRPV1 and shift its activation threshold towards normal body temperature (M. J. Caterina and D. Julius, 2001; M. Tominaga and M. J. Caterina, 2004). While TRPV1 knock-out mice have a normal gross behaviour, they also have some nociceptive deficits expressed through decreased paw licking for example in response to intraplantar capsaicin injection, or impaired thermal hypersensitivity following spinal nerve ligation (M. J. Caterina and D. Julius, 2001). Several potent and selective TRPV1 antagonists are available and demonstrated efficacy in some preclinical models of inflammatory, visceral and neuropathic pain but the TRPV1 antagonist SB-705498 only mildly increased heat pain tolerance in a model of UVB-evoked inflammation in a preliminary human clinical study (B. A. Chizh et al., 2007). This could suggest that a single

drug target is not likely to be the panacea to the heterogeneity of pain states seen in humans. However, by targeting different receptors at the same time, depending on the pain mechanisms involved, the chances of successful outcomes would be likely to increase.

These include the sodium channel NaV 1.8 whose expression is limited to nociceptive primary afferent neurones would be a target of choice in pain treatment (A. N. Akopian et al., 1996; L. Djouhri et al., 2003).

The NMDA receptor, playing a central role in learning and memory through long-term potentiation as well as wind-up (A. H. Dickenson and A. F. Sullivan, 1987) and central sensitisation (C. J. Woolf and S. W. Thompson, 1991) has also long been a target for the treatment of various neurological diseases and pathological pain states. However, until now, NMDA receptor antagonists such as the infamous ketamine were accompanied by unacceptable side-effects (ataxia, hallucinations in addition to analgesia for example), due to the widespread distribution of NMDA receptors and their role in physiological NMDA signalling. However, the NR2B subunit with expression patterns restricted to the forebrain and a very low expression in the cerebellum and in the spinal cord, suggesting a preferential presynaptic location on primary afferent fibres, coupled with the development of NR2B subunit selective antagonists effective on preclinical models of neuropathy with no motor impairment (S. Boyce et al., 1999) could prove to be an interesting target.

Similarly to opioids, cannabinoids, a group of substances that are structurally related to tetrahydrocannabinol (THC) present in *Cannabis sativa*, have been used for centuries to treat pain. However they also have significant physical and psychological side-effects, which limits their use as analgesics. The discovery of specific cannabinoid receptors and their potential pharmacological modulation increased the interest in cannabinoids and endogenous cannabinoids (endocannabinoids), which by acting in the periphery, in the spinal cord and in the brain, for example through the CB1 receptor expressed in the PAG-RVM modulatory system could prove to be useful in pain treating some pain conditions (See reviews by A. G. Hohmann, 2002; J. M. Walker and S. M. Huang, 2002), and maybe in advanced stages of OA.

### 8.4.3 *Combination therapies*

It is quite interesting to notice that since its isolation as the active ingredient of opium in the early 1800s, morphine has remained the gold standard in severe pain conditions, as reminded by the WHO pain relief ladder. In 200 years of research and development, morphine derivatives have been created in order to facilitate its administration and release and to decrease its side-effects, but no new treatment or new drug family has emerged as being more efficient, despite the discovery of many of the molecular actors responsible for pain generation and transmission. This fact could make us think that, on a short-term point of view, some useful pain relief tools may already be in our hands and would just need to be optimised or combined. As can be seen from my data, morphine and gabapentin had different pharmacological profiles on the treatment of OA. This also happens clinically, and the idea of combining both drugs was investigated in human patients, in a high quality clinical trial looking by Gilron et al. (2005) looking at neuropathic pain relief, showing better analgesia with lower doses of each drug, with fewer adverse effects. Indeed, at the doses used, which were not very high, each drug had side-effects such as constipation and diarrhoea for morphine and sedation and dry mouth feelings for gabapentin. The idea was to decrease combine both drugs, enabling the use of lower doses of each, and consequently fewer side-effects, which would act on numerous different molecular targets both in the peripheral and central nervous systems. Again, it is unlikely that one drug, having an effect on one of these targets, could be the panacea for total pain relief. Conversely, two or three drugs could work in synergy on different targets and provide improved pain relief.

The use of drugs association and doses following evidence-based medicine, available for example through the Bandalier website should be encouraged. The Oxford league table of analgesic efficacy provided as Figure 8.8 below shows for example that paracetamol at the dose of 500 mg and celecoxib (a selective COX-2 inhibitor) at 200 mg have a NNT of 3.5 for acute pain relief (50% pain relief over 4-6 hours compared to placebo), whilst for ibuprofen 200 mg the NNT is 2.7 and for paracetamol 1000 mg + codein 60 mg, it decreases to 2.2.



**The 2007 Oxford league table of analgesic efficacy  
(at least 3 trials or 200 patients)**

Numbers needed to treat are calculated for the proportion of patients with at least 50% pain relief over 4-6 hours compared with placebo in randomised, double-blind, single-dose studies in patients with moderate to severe pain. Drugs were oral, unless specified, and doses are milligrams. Shaded rows are intramuscular administration

Analgesic and dose (mg)	Number of patients in comparison	Percent with at least 50% pain relief	NNT	Lower confidence interval	Higher confidence interval
Etoricoxib 180/240	248	77	1.5	1.3	1.7
Etoricoxib 120	500	70	1.6	1.5	1.8
Diclofenac 100	545	69	1.8	1.6	2.1
Celecoxib 400	298	52	2.1	1.8	2.5
Paracetamol 1000 + Codeine 60	197	57	2.2	1.7	2.9
Rofecoxib 50	675	54	2.3	2.0	2.6
Aspirin 1200	279	61	2.4	1.9	3.2
Ibuprofen 400	5456	55	2.5	2.4	2.7
Oxycodone IR 10 + Paracetamol 650	315	66	2.6	2.0	3.5
Diclofenac 25	502	53	2.6	2.2	3.3
Ketorolac 10	790	50	2.6	2.3	3.1
Naproxen 400/440	197	51	2.7	2.1	4.0
Piroxicam 20	280	63	2.7	2.1	3.8
Lumiracoxib 400	370	48	2.7	2.2	3.5
Naproxen 500/550	784	52	2.7	2.3	3.3
Diclofenac 50	1296	57	2.7	2.4	3.1
Ibuprofen 200	3248	48	2.7	2.5	2.9
Pethidine 100 (intramuscular)	364	54	2.9	2.3	3.9
Tramadol 150	561	48	2.9	2.4	3.6
Morphine 10 (intramuscular)	946	50	2.9	2.6	3.6
Naproxen 200/220	202	45	3.4	2.4	5.8
Ketorolac 30 (intramuscular)	359	53	3.4	2.5	4.9
Paracetamol 500	561	61	3.5	2.2	13.3
Celecoxib 200	805	40	3.5	2.9	4.4
Ibuprofen 100	495	36	3.7	2.9	4.9
Paracetamol 1000	2759	46	3.8	3.4	4.4
Paracetamol 600/650 + Codeine 60	1123	42	4.2	3.4	5.3
Paracetamol 650 + Dextropropoxyphene (65 mg hydrochloride or 100 mg napsylate)	963	38	4.4	3.5	5.6
Aspirin 600/650	5061	38	4.4	4.0	4.9
Paracetamol 600/650	1886	38	4.6	3.9	5.5
Ibuprofen 50	316	32	4.7	3.3	8.0
Tramadol 100	882	30	4.8	3.8	6.1
Tramadol 75	563	32	5.3	3.9	8.2
Aspirin 650 + Codeine 60	598	25	5.3	4.1	7.4
Paracetamol 300 + Codeine 30	379	26	5.7	4.0	9.8
Tramadol 50	770	19	8.3	6.0	13.0
Codeine 60	1305	15	16.7	11.0	48.0
Placebo	>10,000	18	N/A	N/A	N/A

Figure 8.8. The 2007 Oxford league table of analgesic efficacy (based on at least 3 clinical studies or 200 patients minimum). Source [www.ebandolier.com](http://www.ebandolier.com)

#### 8.4.4 *Towards cartilage repair?*

Another potential treatment for OA would be anything enabling cartilage repair and thus not only pain relief but also disease modification. These drugs would surely be much more efficient at alleviating pain and have better long-term prognosis. Although there is controversy about the real possibility of modifying the disease *per se*, new agents entering the composition of normal cartilage such as glycosaminoglycans like glucosamine sulphate, and proteoglycans like chondroitin sulfate have recently flourished in health food shops multivitamin sections, claiming to reinforce joints, to be “chondroprotectors” i.e. to protect cartilage and sometimes even to decrease pain associated with OA. The term chondroprotector may not be adequate as this effect has yet to be shown; there is more evidence from high quality clinical trials for significant pain relief for both compounds in severe OA patients, and even a decrease in joint space narrowing (F. Richy et al., 2003; S. Owens et al., 2004).

The possibility of producing cartilage *in vitro* from various types of cells such as stem cells before implanting it into a damaged joint could be a cure one day, with the advantage of autologous grafts (taken from a donor and then reimplanted in the same individual) not being rejected by the immune system.

The different approaches, using a cellular source, scaffolds, and signalling factors that constitute the cartilage tissue triad are shown in Figure 8.9. However, the properties and structure of native cartilage still have not been entirely mimicked by any engineered replacement, whether using chondrocytes, fibroblasts or stem cells cultivated before implantation (C. Chung and J. A. Burdick, 2007). Until those techniques are improved, they will not be able to make their way through the regulatory approval nor be available to patients.

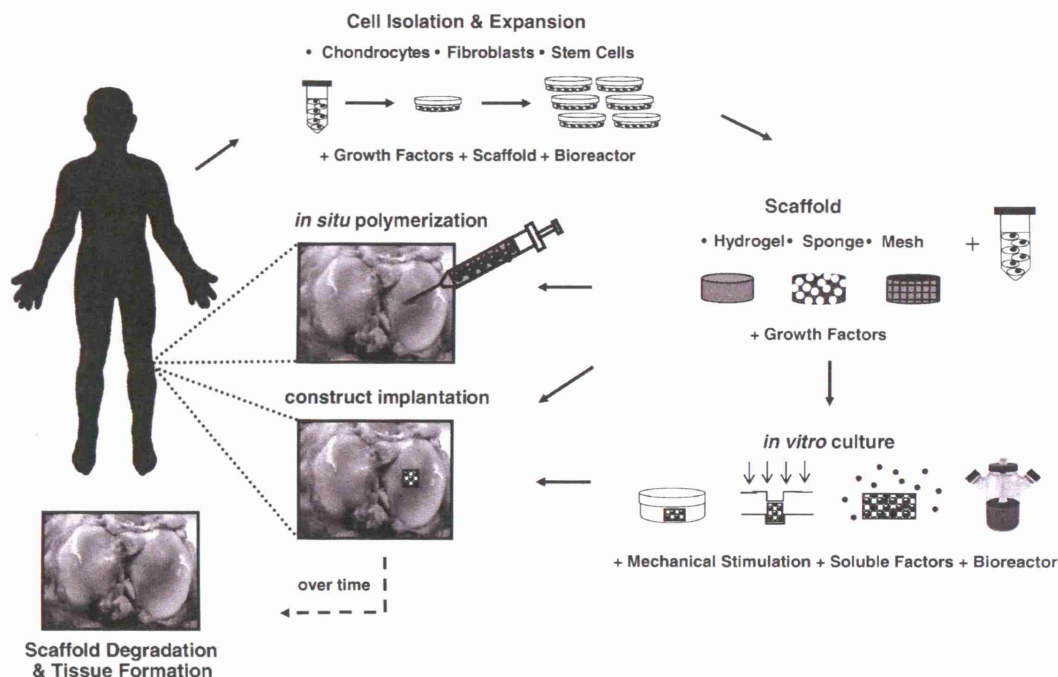


Figure 8.9. General approaches in cartilage tissue engineering, ranging from injectable systems to *in vitro* culture prior to implantation, and numerous biomaterials and culturing methodologies (From C. Chung and J. A. Burdick, 2007).

## 8.5 Usefulness of the model and future studies

As mentioned above, new pain treatment will have to be adapted to each specific pain condition. Whether it is a new analgesic drug that will need to be tested, or the role of a specific receptor in pain modulation, preclinical studies, and this model of OA pain for example will always be necessary before the next stage, i.e. human clinical studies. That was the case for example with the AS006 compound, but also for mechanistic studies.

It will be interesting to use the model, not only to test for new potentially analgesic drugs, but also to perform additional immunohistochemical studies and study for example the distribution of TRPV1 and CGRP, which were shown to be upregulated in the L4 DRG 14 days post-MIA injection (J. Fernihough et al., 2005). One difficulty with CGRP however is the quantification of the expression, as a basal level always exists, and it is a change of intensity that will need to be rated.

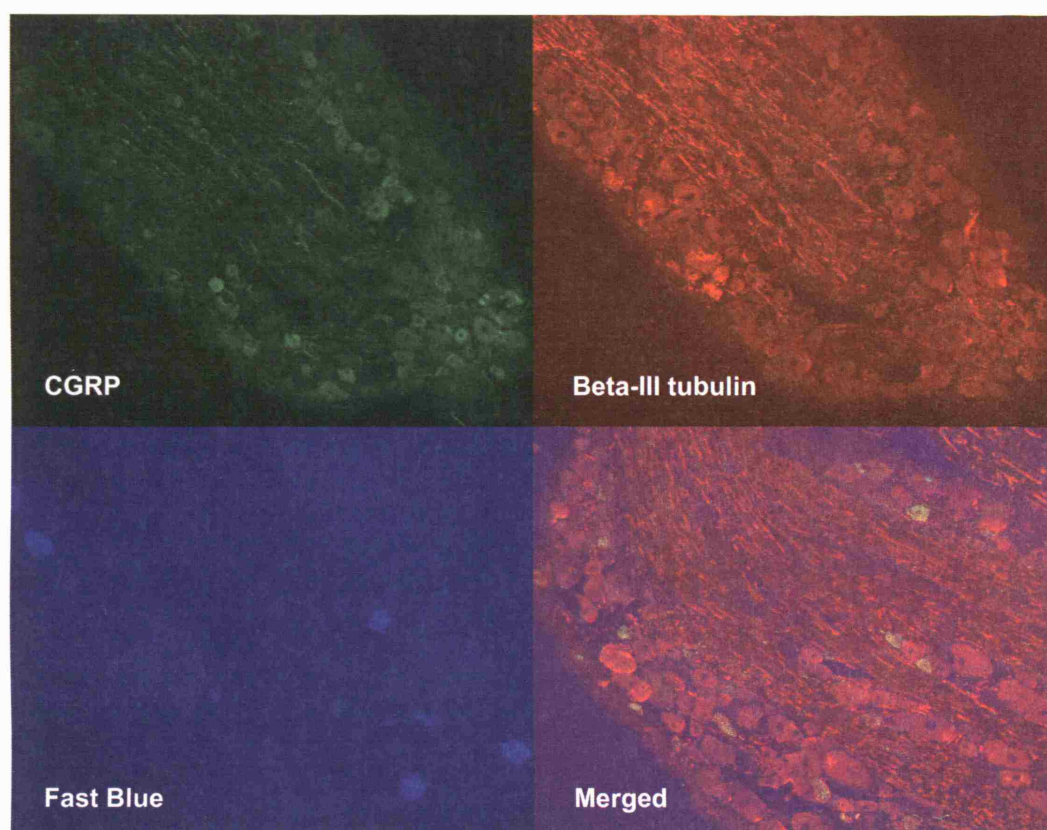


Figure 8.10. CGRP expression in L4 DRG from naive rats. Same antibodies (concentration and species) as before, with rabbit CGRP antibodies used at 1:4000 dilution.

It would also be interesting to study the role of glia in this model of OA pain as it was shown to be an important player in some pain conditions, in particular in neuropathic pain plasticity. Indeed, although most of pain research over the past century focused on neurones, there are about ten glial cells for one neurone in the CNS. After being ignored as they were generally considered primarily to occupy housekeeping roles in the nervous system, there is now evidence of the implication of activated glia in the pathology of neuropathic pain states, by a variety of products, including cytokines (J. A. DeLeo and R. P. Yezierski, 2001; L. R. Watkins et al., 2001; L. R. Watkins and S. F. Maier, 2003).

Three types of glia compose the CNS: astrocytes, oligodendrocytes and microglia. Microglia, a population of macrophages resident in the CNS (5-10 % of total glia), seem to play a central role in neuropathy. Under physiological conditions, microglia are dormant, but they can act as sensors to various stimuli in case of homeostasis perturbation and become activated. They will then undergo changes in morphology from a resting ramified shape into an active amoeboid shape, but also in gene expression, function and number (V. H. Perry, 1994). Various cell surface proteins such as complement receptor-3,

recognised by the antibody OX42, are upregulated. Following their activation by a complex series of signalling systems involving cytokines, chemokines, trophic factors and other neuromodulators, microglia can phagocytose damaged cells, as well as release chemical mediators that can alter neuronal function. In various models of nerve injury such as peripheral nerve injury, microglia in the dorsal horn was shown to correlate with the development of neuropathic pain, implicating p38 MAPK and P2X4 receptors in those pain behaviours (S. X. Jin et al., 2003; M. Tsuda et al., 2003; M. Tsuda et al., 2005). By studying the activation of microglia in DRGs using OX-42 antibody or the expression level of Ib1a (ionised calcium binding adapter molecule 1), another marker of activated microglia (Y. Imai et al., 1996), it would be possible to find out its potential role in this OA pain model.

Additional electrophysiological studies looking at the effects of gabapentin on spinal cord neurones from MIA-treated rats will also be useful, as they will give us some more information about the mechanisms of action of that drug in this model. Indeed gabapentin's ability to decrease neuronal hyperexcitability was shown to be state-dependent as such a decrease was seen following the induction of inflammatory (L. C. Stanfa et al., 1997), neuropathic (E. A. Matthews and A. H. Dickenson, 2002) or cancer-induced bone pain (T. Donovan-Rodriguez et al., 2005) but not in sham rats, which can be explained, at least partly, by the upregulation of the alpha-2-delta-1 subunit of VGCCs seen in models of neuropathy (Z. D. Luo et al., 2001; Z. D. Luo et al., 2002; C. Y. Li et al., 2006). The influence of serotonergic descending facilitation could also be assessed by the direct spinal application of ondansetron, a selective 5-HT<sub>3</sub> receptor antagonist.

Finally, this model is currently used to investigate the role of the alpha-3 subunit of glycine receptors in the MIA model of OA pain by Dr Victoria Harvey in mice. GlyR alpha-3 subunits are mainly expressed in superficial dorsal horn neurones and the release of PGE<sub>2</sub> in inflammatory states can lead to their inhibition, which by causing a decrease in inhibitory post-synaptic currents can result in spinal disinhibition: some GlyR alpha-3 subunit knock-out mice not only presented the lack of inhibition of glycinergic neurotransmission by PGE<sub>2</sub> seen in wild-type mice but also showed a reduction in pain sensitisation induced by spinal PGE<sub>2</sub> injection or peripheral inflammation. Thus, GlyR alpha3 could be playing a major role in central inflammatory pain sensitisation and constitutes a new target in pain treatment (R. J. Harvey et al., 2004). The behavioural and electrophysiological results following the induction of OA using MIA in those mice shall be very interesting and will hopefully complement rat studies.

## **Chapter 9. References**

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**Chapter 10.      Appendix. List of publications**

J.L.Vonsy, J.Ghandehari, A.H.Dickenson (2005).

Behavioural, pharmacological and electrophysiological characterisation of a monosodium iodoacetate - induced model of osteoarthritis.

Program No. 63-P40. 2005 Abstract Viewer. Sydney, Australia: International Association for the Study of Pain.

*11<sup>th</sup> World Pain Congress on Pain, Sydney, Australia 21-26, 2005.*

M.Webber, L.J.Rygh, J.Vonsy, Y.Wong, A.H.Dickenson, S.P.Hunt (2005).

Long Term Potentiation in the Dorsal Horn is Associated with Expression of zif268.

Program No. 1508-P11. 2005 Abstract Viewer. Sydney, Australia: International Association for the Study of Pain

*11<sup>th</sup> World Pain Congress on Pain, Sydney, Australia 21-26, 2005.*

J.L. Vonsy, J. Ghandehari, A.H. Dickenson (2006).

Pharmacological and Electrophysiological studies in the Monosodium Iodoacetate-Induced Model of Osteoarthritis.

Poster Abstract 20

Canadian arthritis network – International conference on preclinical models of osteoarthritis, Montreal, Quebec, Canada, May 18-19, 2006.

Lars Jørgen Rygh, Rie Suzuki, Wahida Rahman, Yuk Wong, Jean Laurent Vonsy, Hardip Sandhu, Mark Webber, Stephen Hunt, Anthony H. Dickenson (2006).

Local and descending circuits regulate long-term potentiation and zif268 expression in spinal neurons.

European Journal of Neuroscience 24 (3), 761–772.

J.L. Vonsy, D.J. Green and A.H. Dickenson (2006).

226 NEURONAL MECHANISMS AND PHARMACOLOGICAL MODULATION OF INFLAMMATION IN THE MONOSODIUM IODOACETATE-INDUCED MODEL OF OSTEOARTHRITIS PAIN.

European Journal of Pain 10 (Supplement 1), S61.

*5<sup>th</sup> Congress of the European Federation of IASP Chapters (EFIC), Istanbul, Turkey, September 13-16, 2006.*